PATENT
Docket No.: **19603/2986 (CRF D-1940B)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Qiu et al.	)	Examiner:
			)	Anne R. Kubelik
Serial No.	:	09/766,348	)	
			)	Art Unit:
Cnfrm. No.	:	7683	)	1638
			)	
Filed	:	January 19, 2001	)	
			)	
For	:	HYPERSENSITIVE RESPONSE INDUCED RESISTANCE IN PLANTS BY SEED TREATMENT	)	

APPEAL BRIEF

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

Pursuant to 37 CFR § 41.37, appellants hereby file their appeal brief along with a petition for a three-month extension of time. The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 14-1138 for the appeal brief filing fee of $510.00, required by 37 CFR § 41.20(b)(2), and the extension of time fee of $1050.00, required by 37 CFR § 1.17(a)(3). Any deficiency/overage can be charged/credited to the same account.

I. REAL PARTY IN INTEREST

Cornell Research Foundation, Inc., as assignee of U.S. Patent Application Serial No. 09/766,348 (referred to herein as "the '348 Application"), is the real party in interest.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences pertaining to the above-identified application.

III. STATUS OF CLAIMS

A. Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 Are Finally Rejected

Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 have been rejected under 35 U.S.C. § 112 (1st para.) for failure to comply with the written description requirement.

Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 have been rejected under 35 U.S.C. § 112 (1st para.) for failure to comply with the enablement requirement.

B. Claims 1-40, 42-48, 52, 54-57, 62-68, 72, 74, 78, 79, 81, 83, and 85 Have Been Canceled

Claims 1-40, 42-48, 52, 54-57, 62-68, 72, 74, 78, 79, 81, 83, and 85 have been canceled.

C. No Claims Stand Allowed

No claims stand allowed.

D. Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 Are On Appeal

The decision of the examiner finally rejecting claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 is appealed. These claims, in their currently pending form, are set forth in the attached **Claims Appendix**.

IV. STATUS OF AMENDMENTS

There are no amendments pending.

V. SUMMARY OF CLAIMED SUBJECT MATTER

This application contains three independent claims—claims 41, 61, and 75.

Claim 41 is directed to a method of imparting pathogen resistance to plants by providing a transgenic plant seed transformed with a transgene containing a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein (page 11, line 31 to page 12, line 5, and page 13, lines 1-4 of the '348 Application). The method also involves planting the transgenic plant seed in soil (page 12, lines 2-3, and page 13, lines 4-5 of the '348 Application)

and propagating a plant from the planted seed (page 12, lines 3-5, and page 13, lines 5-7 of the '348 Application). In accordance with this method, expression of the hypersensitive response elicitor polypeptide or protein by the plant imparts systemic pathogen resistance to the plant (page 12, lines 3-5 and 12 of the '348 Application). The encoded hypersensitive response elicitor polypeptide or protein has an amino acid sequence of SEQ ID NO:1 (page 16, line 9 to page 17, line 25 of the '348 Application), SEQ ID NO:3 (page 18, line 51 to page 20, line 10 of the '348 Application), SEQ ID NO:5 (page 21, line 24 to page 22, line 35 of the '348 Application), or SEQ ID NO:7 (page 23, line 38 to page 24, line 46 of the '348 Application). The transgene also contains a promoter that is not pathogen-inducible (page 36, lines 17-21 of the '348 Application). The promoter is operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein (page 36, lines 14-21 of the '348 Application).

Claim 61 is directed to a method of imparting pathogen resistance to plants by transforming a plant with a transgene containing a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein (page 11, line 31 to page 12, line 5, page 13, lines 1-4, page 33, line 31, and page 36, lines 9-30 of the '348 Application). The transforming of the plant provides for expression of the hypersensitive response elicitor polypeptide or protein that imparts systemic pathogen resistance to the plant (page 12, lines 3-5 and 12, page 33, line 31, and page 36, lines 9-30 of the '348 Application). The encoded hypersensitive response elicitor polypeptide or protein has an amino acid sequence of SEQ ID NO:1 (page 16, line 9 to page 17, line 25 of the '348 Application), SEQ ID NO:3 (page 18, line 51 to page 20, line 10 of the '348 Application), SEQ ID NO:5 (page 21, line 24 to page 22, line 35 of the '348 Application), or SEQ ID NO:7 (page 23, line 38 to page 24, line 46 of the '348 Application). The transgene also contains a promoter that is not pathogen-inducible (page 36, lines 17-21 of the '348 Application). The promoter is operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein (page 36, lines 14-21 of the '348 Application).

Claim 75 is directed to a transgenic plant produced by a process which involves transforming a plant with a transgene containing a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein (page 11, line 31 to page 12, line 5, page 13, lines 1-4, page 33, line 31, and page 36, lines 9-30 of the '348 Application). The transforming of the plant provides for expression of the hypersensitive response elicitor polypeptide or protein that imparts systemic pathogen resistance to the plant (page 12, lines 3-5 and 12, page 33, line 31, and page

36, lines 9-30 of the '348 Application). The encoded hypersensitive response elicitor polypeptide or protein has an amino acid sequence of SEQ ID NO:1 (page 16, line 9 to page 17, line 25 of the '348 Application), SEQ ID NO:3 (page 18, line 51 to page 20, line 10 of the '348 Application), SEQ ID NO:5 (page 21, line 24 to page 22, line 35 of the '348 Application), or SEQ ID NO:7 (page 23, line 38 to page 24, line 46 of the '348 Application). The transgene also contains a promoter that is not pathogen-inducible (page 36, lines 17-21 of the '348 Application). The promoter is operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein (page 36, lines 14-21 of the '348 Application).

VI. GROUNDS OF REJECTION TO BE REVIEWED

Whether claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 are properly rejected under 35 U.S.C. § 112 (1st para.) for failure to satisfy the written description requirement, where the present application clearly teaches the use of non-inducible promoters (including constitutive promoters).

Whether claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 are properly rejected under 35 U.S.C. § 112 (1st para.) for failure to satisfy the enablement requirement, where the present application clearly enables the use of non-inducible promoters (including constitutive promoters).

VII. ARGUMENT

A. Applicable Law—35 U.S.C. § 112 (1st paragraph)

Under 35 U.S.C. § 112 (1st para.), the specification shall contain a written description of the invention . . . in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains . . . to make and use the same . . . .

The "written description" requirement under 35 U.S.C. § 112 (1st para.) has been held to be distinct from the "enablement" requirement of this same section. *See Vas-Cath v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). The purpose of the "written description" requirement is to ensure that the inventor had possession of the invention claimed at the time the application was filed. *Id.* To achieve this, the application must in some manner describe the invention with all its claimed limitations. *See Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997); *In re Wertheim*, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1979). If new matter is added to the claims, the

claims may be subject to rejection under the written description requirement of 35 U.S.C. § 112 (1st para.). *In re Rasmussen*, 650 F.2d 1212, 211 USPQ 323 (CCPA 1981).

Determining whether the description requirement is met must be done on a case-by-case basis and is a question of fact. *In re Wertheim*, 541 F.2d at 262, 191 USPQ at 96 (CCPA 1976). The description, as filed, is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). In instances in which an explicit limitation in a claim "is not present in the written description whose benefit is sought[,] it must be shown that a person of ordinary skill would have understood, at the time the patent application was filed, that the description requires that limitation." *Hyatt v. Boone*, 146 F.3d 1348, 1353, 47 USPQ2d 1128, 1131 (Fed. Cir. 1998)).

To satisfy the enablement requirement of 35 U.S.C. § 112 (1st para.), the applicant must provide sufficient information about the claimed invention that a person of ordinary skill in the field of the invention can make and use it without undue experimentation. *In re Hogan*, 559 F.2d 595, 605, 194 USPQ 527, 536 (CCPA 1977). The amount of skill required to produce the claimed results cannot be unduly extensive, considering the level of unpredictability in the technology. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970).

B. The Rejection of Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 Under 35 U.S.C. § 112 (1st para.) for Failure to Satisfy the Written Description Requirement Is Improper

The examiner has taken the position that neither the specification nor the originally filed claims provide support for the phrase "promoter that is not pathogen-inducible" (as recited in pending claims 41, 61, and 75). For the reasons set forth below, this rejection is improper. More than sufficient written descriptive support exists in the present application for the recited claim language at issue.

The present application generally describes recombinant DNA procedures and materials that can be used to express a particular hypersensitive response elicitor in various types of host cells, including plant cells. This description appears at page 29, line 31 to page 33, line 32. The preparation of transgenic plant seeds is also described at page 36, line 6 to page 37, line 12. Specifically, the following passage appears in the present application at page 36, lines 17-21:

> ***As is conventional in the art***, such transgenic plants would contain suitable vectors with ***various promoters*** including pathogen-induced promoters, and other components needed for transformation, transcription, and, possibly, translation.

(emphasis added). The clear meaning of this language is that "various promoters" can be used to make the claimed transgenic plants and practice the claimed methods.

One type of promoter that falls within the class of "various promoters" is said to be "pathogen-induced promoters." However, the above passage clearly does not limit the "various promoters" to such "pathogen-induced promoters;" pathogen-induced promoters are one example of suitable promoters. In the universe of "various promoters" where "pathogen-induced promoters" are an example, the rest of that universe of "various promoters" must, as a simple matter of logic, be the claimed non-pathogen-inducible promoters. This is entirely consistent with the knowledge that those skilled in the art of transgenic plants would have possessed at the time the present invention was made.

At the time the present invention was made, one of ordinary skill in the art was well aware of the use of constitutive and other non-inducible promoters for transforming plants. *See* Koncz et al., "The Opine Synthase Genes Carried by Ti Plasmids Contain All Signals Necessary for Expression in Plants," *EMBO J.* 2(9):1597-1603 (1983) (referred to herein as "Koncz") (attached hereto as Exhibit 1); U.S. Patent No. 5,034,322 to Rogers et al. (referred to herein as "Rogers '322") (attached hereto as Exhibit 2); and U.S. Patent No. 5,352,605 to Fraley et al. (referred to herein as "Fraley '605") (attached hereto as Exhibit 3). Thus, the phrase "various promoters" in the specification would have been understood by those skilled in the art to encompass, besides pathogen-induced promoters, promoters that are *not* pathogen-inducible (e.g., constitutive promoters).

Koncz was published over 17 years before the filing of the present application, and identifies the nopaline synthase ("NOS") promoter from *Agrobacterium tumefaciens*. As described in more detail below, at the time the present invention was made it was well known that the NOS promoter had been used successfully to transform plant cells with chimeric genes. It is well known in the art that the NOS promoter is *not* a pathogen-induced promoter, but rather is a constitutive promoter. Thus, the NOS promoter qualifies as a promoter that is *not* pathogen-inducible.

Rogers '322 issued as a U.S. patent on July 23, 1991, nearly 10 years before the filing of the present application. Rogers '322 describes chimeric genes that are capable of being

expressed in plant cells (col. 7, lines 18-20). These chimeric genes are said to have been used to create antibiotic-resistant plant cells and as being useful for creating herbicide-resistant plants and plants that contain mammalian polypeptides (Abstract; col. 7, lines 59-64; col. 9, lines 22-25). In a preferred embodiment, the chimeric genes are described as including the NOS promoter from *Agrobacterium tumefaciens* (col. 7, lines 21-29; col. 9, lines 16-17). Rogers '322 also states that "[o]ther suitable promoter regions may be derived from genes which exist naturally in plant cells" (col. 7, lines 29-31). For example, in other preferred embodiments, Rogers '322 teaches making chimeric genes using a promoter region taken from a gene which naturally exists in soybean (i.e., the gene in soybean that codes for the small subunit of ribulose-1,5-bis-phosphate carboxylase) (col. 16, line 48 to col. 18, line 43).

Fraley '605 issued as a U.S. patent on October 4, 1994, over six years before the filing of the present application. Fraley '605 describes chimeric genes for transforming plant cells using viral promoters (col. 3, lines 21-23). In a particular embodiment, Fraley '605 describes using the 35S promoter or the 19S promoter from cauliflower mosaic virus ("CaMV") to make chimeric genes that have been proven to be expressed in plant cells (col. 3, lines 26-37; col 4, line 1 to col. 8, line 62). It is well known in the art that the 35S and 19S promoters are *not* pathogen-induced promoters, but rather are constitutive promoters. Thus, the 35S and 19S promoters qualify as promoters that are *not* pathogen-inducible. Fraley '605 also described using the NOS promoter for constructing chimeric genes for transforming plants (col. 8, line 66 to col. 13, line 51).

Thus, Koncz, Rogers '322, and Fraley '605 constitute strong evidence that non-pathogen-inducible promoters were well known in the art, and known to be useful in preparing transgenic plants or plant tissues. Because these non-pathogen-inducible promoters were well known in the art, and because of the description provided in the present application, the present application clearly intended to cover not just the use of pathogen-inducible promoters, but also various other types of promoters including non-pathogen-inducible promoters.

In making the final rejection, the examiner states that above-quoted page 36, lines 17-21 of the present application shows that "at the time of filing, the only promoters contemplated were pathogen-induced promoters or promoters in general, which included pathogen-induced ones" (page 3 of the Final Office Action, mailed February 26, 2007). Appellants submit that this does not comport with what one of ordinary skill in the art would understand from reading the specification and is an unduly narrow view of the above-quoted

passage. As noted above, the specification teaches making transgenic plants from a genus of "various promoters," with one example being pathogen-induced promoters. Given the knowledge in the art that non-pathogen-inducible promoters are useful in transgenic plants, one of ordinary skill in the art would not simply construe the specification as only teaching the use of pathogen-inducible promoters or promoters generally. Having taught that pathogen-induced promoters are just an example of suitable "various promoters," the other promoters which would constitute suitable various promoters would have to be the well known non-pathogen-inducible promoters.

The examiner's position in the Final Office Action, mailed February 26, 2007, that the specification's recitation of "various promoters including pathogen-induced promoters" does not provide support for promoters *other than* pathogen-inducible promoters (pages 2-3 of the Final Office Action, mailed the February 26, 2007) is even further off-target. Apparently, in the examiner's view, the specification teaches *only* using pathogen-inducible promoters to transform plants with the hypersensitive response elicitors of the present application (*Id.*). In particular, the examiner asserts that, "[a]t the time of filing, the only promoters contemplated were pathogen-induced promoters or promoters in general, which included pathogen-induced ones" (*Id.*). Appellants completely disagree for all of the reasons noted above. Nowhere does the specification limit the claimed promoter to *only* a pathogen-inducible promoter.

For the foregoing reasons, it is submitted that appellants were in possession of the claimed invention at the time they filed the present application. Therefore, the rejection of claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 under 35 U.S.C. § 112 (1st para.) for failure to satisfy the written description requirement is improper and should be withdrawn.

C. The Rejection of Claims 41, 49-51, 53, 58-61, 69-71, 73, 75-77, 80, 82, and 84 Under 35 U.S.C. § 112 (1st para.) for Failure to Satisfy the Enablement Requirement Is Improper

The enablement requirement of 35 U.S.C. § 112 (1st para.) has been interpreted to require that the claimed invention be enabled so that any person skilled in the art can make and use the invention without undue experimentation. *See In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). The disclosure need not teach, and preferably omits, what is well known in the art. *See In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991).

The examiner has taken the position that the application does not enable using a non-pathogen-inducible promoter for transgenic expression of HR elicitors in plants (Final Office Action, mailed February 26, 2007, page 5). The examiner's rationale is that, at the time of filing, constitutive expression of HR elicitors in plants was considered lethal. To support this view, the examiner cites the following references: (i) U.S. Patent No. 5,850,015 to Bauer et al. (referred to herein as "Bauer") (attached hereto as Exhibit 4); (ii) U.S. Patent No. 6,174,717 to Beer et al. (referred to herein as "Beer") (attached hereto as Exhibit 5); and (iii) Tampakaki et al., "Elicitation of Hypersensitive Cell Death by Extracellularly Targeted $HrpZ_{Psph}$ Produced In Planta," *Molecular Plant-Microbe Interactions* 13:1366-1374 (2000) (referred to herein as "Tampakaki") (attached hereto as Exhibit 6). In view of these references, the examiner has alleged that, until the year 2000 (the publication date of Tampakaki), the prevalent view in the art was that constitutive expression of HR elicitors was lethal to the plants. Appellants respectfully disagree.

With respect to Bauer and Beer, appellants assert that these references do not accurately represent the state of the art at the time of filing of the present application. Bauer was originally filed on *June 7, 1995*, as U.S. Patent Application Serial No. 08/484,358. Beer is a patent whose original disclosure was filed on *July 1, 1992*, as U.S. Patent Application Serial No. 07/907,935, now abandoned. Therefore, Bauer and Beer were filed well before the December 3, 1997, filing date of the present application. Thus, based on their filing dates alone, Bauer and Beer cannot be viewed as accurately representing the state of the art at the time of filing the present application.

Compared to the knowledge at the time Bauer and Beer were filed, much more information was available regarding the constitutive expression of HR elicitors in plants at the time of the filing of the present application. As mentioned previously herein, the use of constitutive promoters and other non-pathogen-inducible promoters in transforming plants was well known in December 1997. In addition to the teaching contained in the present application, this view is supported by the experimental data of record in this case, specifically in the Declaration of Zhong-Min Wei Under 37 C.F.R. § 1.132 (dated August 11, 2004) (referred to herein as the "Second Wei Declaration") (submitted with the Amendment dated August 13, 2004) (attached hereto as Exhibit 7). The Second Wei Declaration presents data of the transformation of *Arabidopsis* and tobacco plants with a gene construct containing the *hrpN* gene operatively coupled to the NOS promoter (*see* Second Wei Declaration ¶¶ 25-30). The Second

Wei Declaration states that "[t]he NOS promoter is considered a *weak constitutive promoter*" (Second Wei Declaration ¶ 26) (emphasis added). The data shows that the constitutive expression of HrpN using the NOS promoter was not lethal to the transgenic plants, and that the transgenic plants exhibited pathogen resistance (*see* Second Wei Declaration ¶¶ 28-30).

The examiner quotes Tampakaki as stating that it was "expect[ed] that endogenously produced harpin *may* be lethal to the plant (page 1367, left column, paragraph 4) (emphasis added). This is far from a definitive statement of the state of the art. Nowhere does Tampakaki state that it was the prevalent view or well known in the art that constitutive expression of harpin in plants would necessarily result in plant death. Further, nowhere does Tampakaki teach or suggest that it was the view that *only* pathogen-induced promoters could be used for transforming plants with harpin genes. Instead, at the time of filing of the present application, appellants assert that it would have been reasonable for one of ordinary skill in the art to conclude that using a constitutive promoter (such as the NOS promoter) to transgenically express HR elicitors in plants would not be lethal to the plants. In fact, the vector used by Tampakaki was not a *pathogen*-inducible promoter, but rather a chemical-inducible expression system (i.e., inducible by tetracycline) (*see* Tampakaki, at pages 1367, left column, and 1373, left column).

The examiner states that, "[g]iven the state of the art at the time of filing, use of non-inducible promoters would need to be taught by the specification" (Final Office Action, mailed February 26, 2007, at page 7). Appellants respectfully submit that the use of such promoters is indeed taught by the specification at page 36, lines 17-21. However, the examiner seems to take the view that, in this case, an adequate teaching would require "working examples in which a plant was transformed with a construct comprising a nucleic acid encoding a hypersensitive response elicitor (harpin) of SEQ ID NO:1, 3, 5, or 7 and a non-pathogen inducible promoter" (Final Office Action, mailed February 26, 2007, at page 7). Appellants disagree.

At the time of filing, the basic techniques and components required to transform a plant with a "foreign" gene were well known in the art. This is evidenced by the teachings of Koncz, Bauer, Beer, Fraley, and Rogers. The current claims specify the use of a non-pathogen inducible promoter. At the time of filing, the skilled artisan would have easily determined the types of promoters that would fall into this category of promoters. In other words, such a selection would not have required undue experimentation to select a non-pathogen inducible

promoter that could be used to transform plants. *See* Koncz, Fraley, and Rogers. Finally, evidence introduced by the Second Wei Declaration confirms that a constitutive promoter, the NOS promoter, is effective and non-lethal (*see* Second Wei Declaration ¶¶ 28-30).

For these reasons, appellants respectfully submit that the rejection of claims 41-47, 49-54, 58-73, 75-77, and 80-85 for lack of enablement is improper and should be withdrawn.

VIII. CONCLUSION

In view of the foregoing, it is clear that the rejections of the claims under 35 U.S.C. § 112 (1st para.) cannot be sustained. Accordingly, the rejections should be reversed.

Respectfully submitted,

Dated: January 28, 2008

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IX. CLAIMS APPENDIX

41. A method of imparting pathogen resistance to plants, the method comprising:

providing a transgenic plant seed transformed with a transgene comprising a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein comprising an amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 and a promoter that is not pathogen-inducible, the promoter being operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein;

planting the transgenic plant seed in soil; and

propagating a plant from the planted seed, whereby expression of the hypersensitive response elicitor polypeptide or protein by the plant imparts systemic pathogen resistance to the plant.

49. The method according to claim 41, wherein the plant is selected from the group consisting of dicots and monocots.

50. The method according to claim 49, wherein the plant is selected from the group consisting of rice, wheat, barley, rye, oats, cotton, sunflower, canola, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

51. The method according to claim 49, wherein the plant is selected from the group consisting of rose, *Saintpaulia,* petunia, *Pelargonium*, poinsettia, chrysanthemum, carnation, and zinnia.

53. The method according to claim 41 further comprising:

applying the hypersensitive response elicitor polypeptide or protein to the plant to enhance the plant's pathogen resistance.

58. A plant produced by the method of claim 41.

59. A transgenic plant seed from the plant produced by the method of claim 41, wherein the transgenic plant seed comprises the transgene.

60. A plant propagule from the plant produced by the method of claim 41.

61. A method of imparting pathogen resistance to plants, the method comprising:

transforming a plant with a transgene comprising a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein comprising an amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 and a promoter that is not pathogen-inducible, the promoter being operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein, whereby said transforming provides for expression of the hypersensitive response elicitor polypeptide or protein that imparts systemic pathogen resistance to the plant.

69. The method according to claim 61, wherein the transgenic plant is selected from the group consisting of dicots and monocots.

70. The method according to claim 69, wherein the plant is selected from the group consisting of rice, wheat, barley, rye, oats, cotton, sunflower, canola, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

71. The method according to claim 69, wherein the plant is selected from the group consisting of rose, Saintpaulia, petunia, Pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

73. The method according to claim 61, further comprising:

applying the hypersensitive response elicitor polypeptide or protein to the transgenic plant to enhance the plant's pathogen resistance.

75. A transgenic plant produced by a process comprising:

transforming a plant with a transgene comprising a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein comprising an amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 and a promoter that is not pathogen-inducible, the promoter being operatively coupled to the DNA molecule encoding the hypersensitive response elicitor polypeptide or protein, whereby said transforming provides for

expression of the hypersensitive response elicitor polypeptide or protein to impart systemic pathogen resistance to the transgenic plant.

76. A transgenic plant seed obtained from the transgenic plant of claim 75, wherein the transgenic plant seed comprises the transgene.

77. A transgenic plant propagule obtained from the transgenic plant of claim 75.

80. The method according to claim 41, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8.

82. The method according to claim 61, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8.

84. The transgenic plant according to claim 75, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8.

X. EVIDENCE APPENDIX

A. **EXHIBIT 1** - Koncz et al., "The Opine Synthase Genes Carried by Ti Plasmids Contain All Signals Necessary for Expression in Plants," *EMBO J.* 2(9):1597-1603 (1983)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action, dated October 29, 2004.

B. **EXHIBIT 2** - U.S. Patent No. 5,034,322 to Rogers et al.

- Discussed in appellants' Amendment Under 37 CFR § 1.116, dated March 29, 2005, and considered by the examiner in the Advisory Action, dated April 20, 2005.

C. **EXHIBIT 3** - U.S. Patent No. 5,352,605 to Fraley et al.

- Discussed in appellants' Amendment Under 37 CFR § 1.116, dated March 29, 2005, and considered by the examiner in the Advisory Action, dated April 20, 2005.

D. **EXHIBIT 4** - U.S. Patent No. 5,850,015 to Bauer et al.

- Relied upon and discussed in the Examiner's Answer, dated November 20, 2006.

E. **EXHIBIT 5** - U.S. Patent No. 6,174,717 to Beer et al.

- Relied upon and discussed in the Examiner's Answer, dated November 20, 2006.

F. **EXHIBIT 6 -** Tampakaki et al., "Elicitation of Hypersensitive Cell Death by Extracellularly Targeted $HrpZ_{Psph}$ Produced In Planta," *Molecular Plant-Microbe Interactions* 13:1366-1374 (2000)

- Relied upon and discussed in the Examiner's Answer, dated November 20, 2006.

G. **EXHIBIT 7 -** Declaration of Zhong-Min Wei Under 37 C.F.R. § 1.132 ("Second Wei Declaration")

- Introduced by appellant on August 13, 2004, and considered by the examiner in the office action, dated October 29, 2004.

XI. RELATED PROCEEDINGS APPENDIX

There are no decisions rendered by a court or the Board on related appeals or interferences.

Exhibit 1 - Koncz et al., *EMBO J.* 2(9):1597-1603 (1983)

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The opine synthase genes carried by Ti plasmids contain all signals necessary for expression in plants

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Signals necessary for *in vivo* expression of Ti plasmid T-DNA-encoded octopine and nopaline synthase genes were studied in crown gall tumors by constructing mutated genes carrying various lengths of sequences upstream of the 5′ initiation site of their mRNAs. Deletions upstream of position −294 did not interfere with expression of the octopine synthase gene while those extending upstream of position −170 greatly reduced the gene expression. The estimated size of the octopine synthase promoter is therefore 295 bp. The maximal length of 5′ upstream sequences involved in the *in vivo* expression of the nopaline synthase gene is 261 bp. Our results also demonstrated that Ti plasmid-derived sequences contain all signals essential for expression of opine synthase genes in plants. Expression of these genes, therefore, is independent of the direct vicinity of the plant DNA sequences and is not activated by formation of plant DNA and T-DNA border junction.

Key words: Agrobacterium tumefaciens/Ti plasmids/opine synthase genes/promoter regions

Introduction

Crown gall, a neoplastic disease of dicotyledonous plants, develops after infection of wounded tissue with *Agrobacterium tumefaciens* strains carrying large tumor-inducing (Ti) plasmids (Zaenen *et al.*, 1974; Van Larebeke *et al.*, 1974; Watson *et al.*, 1975). A well-defined segment (T-region) of the Ti plasmid is transferred and covalently integrated, without rearrangements, in plant nuclear DNA (Chilton *et al.*, 1977, 1980; Schell *et al.*, 1979; Thomashow *et al.*, 1980; Lemmers *et al.*, 1980; Zambryski *et al.*, 1980; Yadav *et al.*, 1980; Willmitzer *et al.*, 1980). The transferred DNA (T-DNA) is transcribed (Drummond *et al.*, 1977; Willmitzer *et al.*, 1981a; Gelvin *et al.*, 1981) by the host RNA polymerase II (Willmitzer *et al.*, 1981b).

Transformed crown gall cells are capable of autonomous growth in the absence of exogenous phytohormones (Braun, 1956). Moreover, these plant tumors synthesize a variety of low mol. wt. metabolites (termed opines) which are characteristic for Ti plasmid-induced tumors (Bomhoff *et al.*, 1976), and can be specifically metabolized by agrobacteria growing on the incited tumors (Petit *et al.*, 1970; Petit and Tempé, 1978; Schell *et al.*, 1979; Tempé *et al.*, 1980). The Ti plasmids are currently classified into three groups according to the type of opine they induce in the incited tumors as octopine, nopaline or agropine Ti plasmids (Guyon *et al.*, 1980).

The T-DNA in octopine tumors consists of two distinguishable segments: TL-DNA and TR-DNA (Thomashow *et al.*, 1980; De Beuckeleer *et al.*, 1981). TL-DNA, which is essential and sufficient for octopine crown gall formation, codes for eight polyadenylated transcripts, each expressed from an individual promoter (Gelvin *et al.*, 1982; Willmitzer *et al.*, 1982). One of these transcripts (transcript 3) was shown to code directly for the enzyme octopine synthase (Schröder *et al.*, 1981). The nucleotide sequence of this gene was elucidated and both the 5′ and the 3′ ends of the transcript were precisely identified by S1 nuclease mapping (De Greve *et al.*, 1982). The 5′ end of the transcript coding for octopine synthase is located close to the right border of TL-DNA at a distance of 350 – 400 bp. This gene is transcribed from right to left (Willmitzer *et al.*, 1982).

The T-DNA of nopaline Ti plasmids codes for up to 13 polyadenylated transcripts (Bevan and Chilton, 1982; Willmitzer *et al.*, 1983). The region responsible for tumor maintenance is highly homologous between octopine TL-DNA and nopaline T-DNA (Engler *et al.*, 1981). Transcripts and gene functions determined by this conserved 'core' region are common in octopine and nopaline tumors (Joos *et al.*, 1983; Willmitzer *et al.*, 1983). Two different opines were detected in nopaline tumors: agrocinopine (Ellis and Murphy, 1981) and nopaline (Petit *et al.*, 1970). The nopaline synthase gene has been localized by genetic and transcript mapping on *Hind*III fragment 23 of plasmids pTiC58 and pTiT37 (Holsters *et al.*, 1980; Hernalsteens *et al.*, 1980; Joos *et al.*, 1983; Willmitzer *et al.*, 1983). DNA sequencing of *Hind*III fragment 23 localized the nopaline synthase gene (Depicker *et al.*, 1982) and the precise position of the right T-DNA borders within *Hind*III fragment 23 (Zambryski *et al.*, 1982).

To determine whether all signals essential for the expression of the opine synthase genes *in vivo* are located between the 5′ initiation site of the opine genes and the junction site with plant DNA or whether expression of these genes is activated by plant DNA sequences, we constructed octopine and nopaline synthase genes with different lengths of sequences upstream of the 5′ initiation site and reinserted them in the T-DNA of the Ti plasmids. This approach allowed us to delimit which sequences are important for the *in vivo* expression of the octopine and nopaline synthase genes, and to demonstrate that the plasmid-derived sequences contain all signals necessary for expression in plants.

Results

Expression of the octopine synthase gene in nopaline tumors

Construction of intermediate vectors pGV761, pGV762 and pGV763. The precise number of base pairs in the DNA region between the 5′ initiation site of the octopine synthase transcript (De Greve *et al.*, 1982) and the right T-region border sequence (Holsters *et al.*, 1983) has been determined and was found to be 402 (Figure 1a). Therefore, sequences essential for the expression of octopine synthase must either be located in this sequence, or activation of the promoter occurs by junction of the 5′ end of the *ocs* gene with plant

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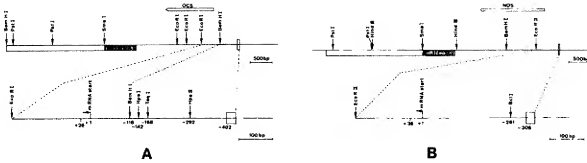

Fig. 1. (A) In the upper part the *Bam*HI fragment 17a and sequences up to the border (white box) are indicated, and the location and transcription polarity of the octopine synthase gene. The white bar shows the homology region between *Bam*HI fragment 17a and the nopaline T-DNA. The hatched portion of the white bar shows the homology region of 750 bp between plasmids pGV761, pGV762, pGV763 and the nopaline Ti plasmid. In the lower part the position of the restriction sites used in this study are indicated with regard to the transcription start of the octopine synthase gene. (B) In the upper part the *Hind*III fragments 23 and 31, and part of the *Hind*III fragment 22, are indicated (Depicker *et al.*, 1980). The position and transcription polarity of the nopaline synthase gene located in *Hind*III fragment 23 and the homology region with *Bam*HI fragment 17a of the octopine Ti plasmid pTiAch5 are shown. In the lower part the position of the *Bcl*I site is indicated with regard to the transcription start of the nopaline synthase gene.

DNA.

To test which of these possibilities is valid, intermediate vectors containing the octopine synthase gene and different lengths of 5'-flanking sequences (respectively −116 bp, −168 bp and −292 bp from the transcription start; Figure 1a) were constructed and introduced into the nopaline Ti plasmid C58. If the first possibility is correct, these constructions should allow us to delimit the sequences involved in the *in vivo* expression of the octopine synthase gene. The different steps in the construction of the intermediate vectors are outlined in Figure 2.

Isolation of co-integrated Ti plasmids. As the homology region between plasmids pGV761, pGV762 and pGV763 (Figure 1), and the nopaline Ti plasmid is only 750 bp, we envisaged, to avoid problems of recombination, using the homology of 1270 bp between the *amp* gene located on pBR322 and the transposon Tn*1*, inserted into the T-DNA of the nopaline Ti plasmid C58 (Joos *et al.*, 1983; Inzé *et al.*, in preparation).

For this purpose, we selected the plasmids pGV3300 and pGV3305. In pGV3300 a Tn*1* is inserted in *Hind*III fragment 23 just outside the nopaline synthase gene, while in pGV3305 the Tn*1* insertion is located in the nopaline synthase gene. The intermediate vectors pGV761, pGV762 and pGV763 were mobilized from *Escherichia coli* to *Agrobacterium* strains GV3101 (pGV3300) and GV3101 (pGV3305) with the helper plasmids R64*drd*11 and pGJ28 (Van Haute *et al.*, 1983). In all cases, Km^R transconjugants were isolated with a frequency of 10^{-6}–10^{-7}. Several co-integrate plasmids resulting from a single cross-over were analyzed by DNA/DNA hybridization to confirm their physical structure (data not shown). Recombination always occurred within the homology region common to pBR322 and Tn*1*.

Properties of the co-integrated plasmids. Sunflower hypocotyls and tobacco W38 plants were inoculated with the *Agrobacterium* strains containing these different co-integrates. The different primary tumor tissues were subsequently analyzed for octopine synthase activity (Otten and Schilperoort, 1978). No octopine synthase activity was detected in sunflower and tobacco tumors induced by the *Agrobacterium* strains containing the co-integrated plasmids pGV2290 (pGV3300::pGV761) and pGV2291 (pGV3305::pGV761). Furthermore, in tumors induced by *Agrobacterium* strains containing the co-integrated plasmids pGV2292 (pGV3300::pGV762) and pGV2293 (pGV3305::pGV762), again no detectable octopine synthase activity could be detected. On the contrary, in sunflower and tobacco tumors induced with *Agrobacterium* strains containing the co-integrated plasmids pGV2294 (pGV3300::pGV763) and pGV2295 (pGV3305::pGV763), octopine synthase activity was detected (Figure 3). The level of activity in these tumors was equal to that found in tumors induced by the *Agrobacterium* strain C58C1 containing an octopine Ti plasmid ($pTiB6S3Tra^C$).

Expression of the nopaline synthase gene in octopine tumors

We have studied the expression of the nopaline synthase gene by a similar approach. DNA sequence analysis showed that the nopaline synthase gene is entirely encoded by the *Hind*III fragment 23 of pTiC58 (Depicker *et al.*, 1982). Furthermore, genomic blotting analysis of nopaline tumor tissues (Lemmers *et al.*, 1980) showed that this *Hind*III-23 fragment is a border fragment. Genomic clones isolated from different nopaline tumor tissues (Zambryski *et al.*, 1980, 1982; Holsters *et al.*, 1982) allowed us to determine the exact end points of the T-DNA in crown gall lines. The right T-DNA/plant DNA border is located only 305 bp (Figure 1b) from the start of the nopaline synthase transcript (Depicker *et al.*, 1982).

Construction and properties of pGV2253 and pGV2254

Construction of intermediate vectors pGV705 and pGV706. To demonstrate that the expression of the nopaline synthase gene is independent of the formation of a junction to plant DNA sequences, and that all sequences involved in the *in vivo* expression of the nopaline synthase gene are present between the start of the mRNA and the end of the T-DNA, we constructed an intermediate vector in which the sequences between the *Hind*III site and the *Bcl*I site (position −261; Figure 1b) of the *Hind*III fragment 23 have been deleted and replaced by the Sm^R gene of R702. This substitution deletes the 22-bp consensus sequence (position −305; Figure 1b) which is found at the ends of nopaline and octopine T-regions, and which might play a key role in the integration of the T-region into the plant genome (Zambryski *et al.*, 1980, 1982; Simpson *et al.*, 1982; Yadav *et al.*, 1982; Holsters *et al.*, 1982, 1983). The construction of the intermediate vector pGV705 is shown in Figure 4.

pGV705 consists of *Eco*RI fragment 12 of pTiAch5 in which the internal *Hind*III-36a fragment has been substituted

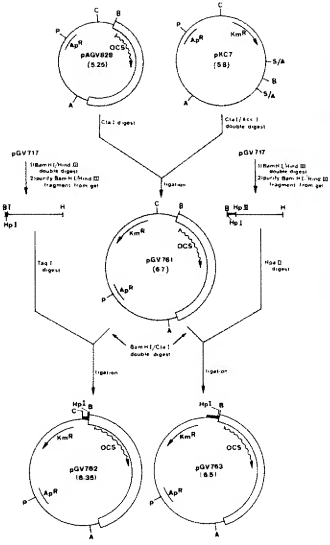

Fig. 2. Construction of intermediate vectors pGV762 and pGV763. The *Acc*I-*Cla*I fragment of pKC7 containing the Km gene was ligated to *Cla*I-digested pAGV828. After ligation and selection on ApKm plates, recombinants were screened for the orientation of the Km-resistant fragment by double digestion with *Cla*I and *Bam*HI. A recombinant plasmid pGV761 was digested with *Bam*HI and *Cla*I, and ligated to the purified *Hind*III-*Bam*HI fragment of pGV717, which contains sequences 5′ upstream of the *Bam*HI site at −116 in the promoter region of the octopine synthase gene (Figure 1; Holsters *et al.*, 1983), digested with either *Taq*I or *Hpa*II. By screening recombinant plasmids for the presence of a *Hpa*I site (Figure 1), pGV762 and pGV763 were obtained. Abbreviations: A, *Acc*I; B, *Bam*HI; C, *Cla*I; H, *Hind*III; Hpl, *Hpa*I; HpII, *Hpa*II; P, *Pst*I; S, *Sal*I; T, *Taq*I.

by the *Hind*III-*Bcl*I fragment of the nopaline *Hind*III fragment 23 joined to the *Bam*HI-*Hind*III fragment of plasmid pR702 containing the Sm^R gene. This *Hind*III fragment inserted in the other orientation in the *Eco*RI fragment I2, is called pGV706.

Isolation of pGV2253 and pGV2254. The intermediate vectors pGV705 and pGV706 were mobilized from *E. coli* to *Agrobacterium* strain GV3000 carrying a transfer-constitutive pTiB6S3 plasmid with the help of the plasmids R64*drd*11 and pGJ28 (Van Haute *et al.*, 1983). Streptomycin-resistant *Agrobacterium* strains were obtained in both cases with a joint transfer and recombination frequency of 10^{-6}. The Sm-resistant transconjugants were tested directly for Km sensitivity. Three percent of the Sm^R transconjugants were Km-sensitive and were double recombinants. The structure of two plasmids pGV2253 and pGV2254 was confirmed by DNA-DNA hybridization (data not shown).

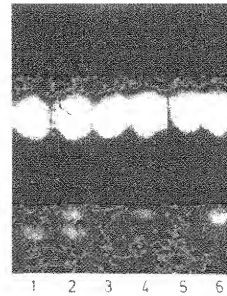

Fig. 3. Detection of octopine in tumors induced with *Agrobacterium* strains containing the mutant plasmids. 2 μl of extracts of tumor tissue before (**lanes 1, 3, 5**) and after (**lanes 2, 4, 6**) 1 h incubation were spotted onto Whatman 3MM paper and subjected to electrophoresis. **Lanes 1** and **2**: extracts obtained from tissue infected with *Agrobacterium* containing pGV2295; **lanes 3** and **4**: extracts obtained from tissue infected with *Agrobacterium* containing pGV2294; **lanes 5** and **6**: extracts obtained from tissue infected with *Agrobacterium* containing pGV2254.

Properties of pGV2253 and pGV2254. *Agrobacterium* strain containing either pGV2253 or pGV2254 were used to incite tumors on tobacco plants. These tumors synthesize nopaline and octopine (Figure 3), but no mannopine or agropine could be detected. This observation indicates that the deletion substitution of the small *Hind*III fragment 36a abolishes the synthesis of mannopine and agropine.

Morover, since the sequences between the end of the nopaline T-DNA (position −305) and the *Bcl*I site (position −261) have been deleted and replaced by the Sm^R gene of pR702, the 5′-flanking region of the nopaline synthase gene in this construction is separated from TR sequences located to the right (in pGV2253) or to the left (in pGV2254), by the Sm^R insert fragment. Therefore, all the sequences involved in the *in vivo* expression of the nopaline gene must lie within the 5′-flanking region between the start of transcription and the *Bcl*I site (position −261).

Discussion

Most of the understanding of the regulatory events controlling gene expression in higher eukaryotes is derived from studies with animal viruses. Several eukaryotic promoters have been examined both by DNA sequencing and by *in vitro* and *in vivo* analysis of mutants. These studies have led to the identification of the so-called Goldberg-Hogness or TATA box, a signal that is involved in the precise positioning of 5′ RNA ends of genes transcribed by RNA polymerase II (Breathnach and Chambon, 1981; Shenk, 1981). Although the TATA box seems to be both necessary and sufficient for

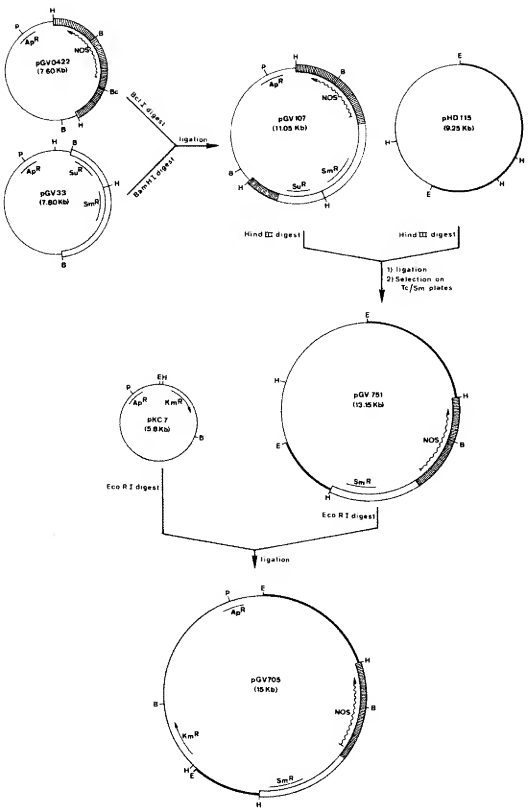

Fig. 4. Construction of the intermediate vector pGV705. Plasmids pGV0422 was linearized with *Bcl*I and ligated to *Bam*H1-digested pGV33. After transformation, recombinants were selected on Ap/Sm plates. One of the recombinants, pGV107, was digested with *Hind*III and ligated to *Hind*III-digested pHD115, containing the *Eco*RI fragment 12 of pTiAch5. After selection on Tc/Sm plates a recombinant, pGV751, was digested with *Eco*RI and ligated to *Eco*RI-digested pKC7, making it possible to use the mobilizing method described by Van Haute *et al.* (1983). Indeed, pGV751, a pACYC184 derivative, cannot be mobilized by pGJ28 and R64*drd*11.

Table 1. Bacterial strains and plasmids

	Antibiotic resistance	Characteristics	Dimension (kb)	Origin
Strains				
E. coli				
K514		*thr leu thi hsd*R		Colson *et al.* (1965)
A. tumefaciens				
GV3101		Rif^R derivative of C58, cured for pTiC58		Van Larebeke *et al.* (1974)
GV3105		Ery^R Cml^R derivative of C58, cured for pTiC58		Holsters *et al.* (1980)
Plasmids				
pKC7	Ap Km	*Hind*III-*Bam*HI of Tn*5* in pBR322	5.8	Rao and Rodgers (1979)
pGV0153	Ap	*Bam*HI-8 of pTiAch5 in pBR322	11.6	De Vos *et al.* (1981)
pGV0201	Ap	*Hind*III-1 of pTiAch5 in pBR322	16.9	De Vos *et al.* (1981)
pGV0422	Ap	*Hind*III-23 of pTiC58 in pBR322	7.6	Depicker *et al.* (1980)
pGV705	Ap Km Sm	*Hind*III fragment containing the *nos* gene and Sm/Sp marker of R702 in *Eco*RI-12	15	This work
pGV706	Ap Km Sm	*Hind*III fragment containing the *nos* gene and Sm/Sp marker of R702 in *Eco*RI-12, but in opposite direction	15	This work
pGV717	Ap	*Hind*III-*Bam*HI fragment of gc1 rGV1-1 in pBR322	5.1	Holsters *et al.* (1983)
pAGV828	Ap	*Bam*HI-*Sma*I of pGV99 in pBR322	5.25	Herrera-Estrella *et al.* (1983)
pGV761	Ap Km	*Cla*I-*Acc*I of pKC7 in pAGV828	6.7	This work
pGV762	Ap Km	*Taq*I-*Bam*HI of pGV717 in pGV761	6.35	This work
pGV763	Ap Km	*Hpa*II-*Bam*HI of pGV717 in pGV761	6.5	This work
pGV33	Ap Sm/Sp Su	3.5 kb *Bam*HI fragment of R702 in pBR322	7.7	J. Leemans
pHD115	Tc	*Eco*RI-12 fragment of pTiAch5 in pACY184	9.25	J. Velten
R702	Km Sm/Sp Tc Su Hg	P-type plasmid	69.0	Hedges and Jacobs (1974)
R64drd11	Tc Sm	Io-type plasmid, transfer-derepressed derivative of R64	109.0	Meynell and Datta (1967)
pGJ28	Km/Nm	Cda^+ Ida^+ ColD replicon carrying ColE1 *mob* and *bom*	9.7	Van Haute *et al.* (1983)
pGV3100	–	pTiC58, derepressed for autotransfer	212	Holsters *et al.* (1980)
pGV3300	Ap	pGV3100::Tn*1*	215	Joos *et al.* (1983)
pGV3305	Ap	pGV3100::Tn*1*	215	D. Inzé
pTiB6S3 Tra^c		pTiB6S3, derepressed for autotransfer	192	Petit *et al.* (1978)

accurate initiation of transcription *in vitro* (Corden *et al.*, 1980; Wasylyk *et al.*, 1980), regions further upstream are required for efficient *in vivo* transcription (Grosschedl and Birnstiel, 1980; Benoist and Chambon, 1980; McKnight *et al.*, 1981; Grosveld *et al.*, 1982; Weiher *et al.*, 1983). Recently, a detailed analysis of the promoter of the herpes simplex thymidine kinase (TK) gene (McKnight and Kingsbury, 1982) resulted in an identification of three essential regions within 105 bp upstream of the RNA initiation site.

In higher plants, on the contrary, little is known about sequence signals controlling gene expression. In octopine and nopaline crown gall tumor tissues, the T-DNA is transcribed by RNA polymerase II (Willmitzer *et al.*, 1981a), and encodes a set of well-defined polyadenylated transcripts. Therefore, the T-DNA genes can serve as models for defining transcriptional and translational control sequences in nuclear, protein-coding plant genes. In a first approach, we have attempted to determine which are the minimal 5' upstream sequences involved in the *in vivo* expression of these opine genes. Deletion of sequences upstream of position −170 of the octopine synthase gene greatly reduces or abolishes the gene expression, while deletion of sequences upstream of position −294 does not interfere with a wild-type level of gene expression. In this sequence of 125 bp an essential region controlling the expression of the octopine synthase gene might be located. Also in the case of the nopaline synthase gene, the 5' sequences downstream of position −261 contain all the information necessary for the *in vivo* expression of this gene. Therefore, the estimated maximum size of the octopine and nopaline synthase gene promoters are 295 bp and 261 bp, respectively. Although the DNA sequences directly involved in the expression of the opine synthase genes in plant cells are not defined in this study, and identification of these sequences could help in the elucidation of the mechanisms of plant cellular gene control, the results described above clearly demonstrate that the expression of octopine and nopaline synthase genes is

determined directly by their 5′ upstream flanking sequences and is independent of the direct vicinity of the plant DNA sequences.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table 1.

Media and culture conditions

Luria broth (LB) and minimal A (minA) media were as described (Miller, 1972). Nitrogen-free medium for the use of octopine or nopaline as sole nitrogen source were as described (Bomhoff *et al.*, 1976). *E. coli* cultures were grown at 37°C and *A. tumefaciens* at 28°C. Antibiotic concentrations used for *E. coli* and *A. tumefaciens* were respectively, carbenicillin (Cb), 100 μg/ml; streptomycin (Sm), 20 μg/ml and 300 μg/ml; spectinomycin (Sp), 50 μg/ml and 100 μg/ml; kanamycin (Km), 25 μg/ml; rifampicin (Rif), 100 μg/ml; erythromycin (Ery), 50 μg/ml for *Agrobacterium*; chloramphenicol (Cml), 25 μg/ml for *Agrobacterium*.

Plasmid isolation

Plasmids were prepared from *E. coli* by density gradient centrifugation in a CsCl-ethidium bromide gradient of cleared SDS lysates (Betlach *et al.*, 1976). For screening of recombinant plasmids, plasmid DNA was obtained from 10 ml cultures as described (Klein *et al.*, 1980).

DNA analysis

Restriction enzyme analysis, agarose gel electrophoresis, conditions for DNA ligation and transformation of competent *E. coli* were as described (Depicker *et al.*, 1980). DNA fragments were extracted from low-gelling agarose gels as described (Wieslander, 1979). Total DNA of Ti plasmid-containing *Agrobacterium* strains was prepared, digested, separated on agarose gel, transferred to nitrocellulose paper, and hybridized against radioactively labeled recombinant plasmids as described (Dhaese *et al.*, 1979).

Induction and culture of crown gall tumors

Sterile 1-month-old tobacco plants (Wisconsin 38 or SR1) were decapitated and infected with freshly grown agrobacteria. Three weeks later, tumors were excised and transferred to hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) containing sucrose (30 g/l) and 0.5 mg/ml HR756 (Hoechst A.G.). The tumor tissues, transferred every month, were usually free of bacteria after three transfers, and were further cultivated on antibiotic-free Murashige and Skoog medium. Sunflower hypocotyl segments were inoculated as described by Petit and Tempé (1978).

Detection of opines in plant tumor tissue

Octopine and nopaline detection. The presence of octopine or nopaline in tumor tissue was tested as described by Leemans *et al.* (1981). Octopine or nopaline synthase activity were determined *in vitro* according to Otten and Schilperoort (1978).

Agropine and mannopine detection. Agropine and mannopine were detected in tumor tissue as described by Leemans *et al.* (1981).

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Exhibit 2 - U.S. Patent No. 5,034,322 to Rogers et al.

United States Patent [19]

Rogers et al.

[11] **Patent Number: 5,034,322**

[45] **Date of Patent: Jul. 23, 1991**

[54] **CHIMERIC GENES SUITABLE FOR EXPRESSION IN PLANT CELLS**

[75] Inventors: **Stephen G. Rogers,** Webster Groves; **Robert T. Fraley,** Glendale, both of Mo.

[73] Assignee: **Monsanto Company,** St. Louis, Mo.

[21] Appl. No.: **333,802**

[22] Filed: **Apr. 5, 1989**

Related U.S. Application Data

[63] Continuation of Ser. No. 793,488, Oct. 30, 1985, abandoned, which is a continuation of Ser. No. 458,414, Jan. 17, 1983, abandoned.

[51] **Int. Cl.**[5] **C12P 21/00;** C12N 15/00; C12N 9/00; C12N 1/20

[52] **U.S. Cl.** **435/172.3;** 435/69.1; 435/183; 435/252.2; 435/252.3; 435/252.33; 435/320.1; 536/27; 935/30; 935/35; 935/36; 935/67

[58] **Field of Search** 435/68, 172.3, 183, 435/252.2, 252.3, 252.33, 320, 320.1, 69.1; 536/27

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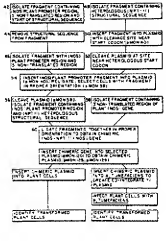
Primary Examiner—Jacqueline Stone
Assistant Examiner—David T. Fox
Attorney, Agent, or Firm—Dennis R. Hoerner, Jr.; Thomas P. McBride; Howard C. Stanley

[57] **ABSTRACT**

This invention relates to chimeric genes which are capable of being expressed in plant cells. Such genes contain (a) a promoter region derived in a gene which is expressed in plant cells, such as the nopaline synthase gene; (b) a coding or structural sequence which is heterologous with respect to the promoter region; and (c) an appropriate 3' non-translated region. Such genes have been used to create antibiotic-resistant plant cells; they are also useful for creating herbicide-resistant plants, and plants which contain mammalian polypeptides.

31 Claims, 27 Drawing Sheets



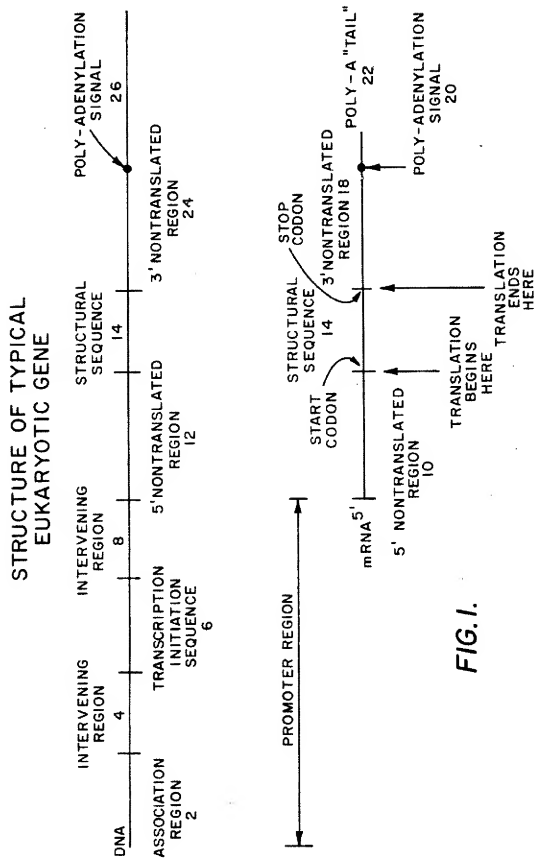

STRUCTURE OF TYPICAL EUKARYOTIC GENE
DNA
ASSOCIATION REGION 2
INTERVENING REGION 4
TRANSCRIPTION INITIATION SEQUENCE 6
INTERVENING REGION 8
5'NONTRANSLATED REGION 12
STRUCTURAL SEQUENCE 14
3'NONTRANSLATED REGION 24
POLY-ADENYLATION SIGNAL 26
PROMOTER REGION
mRNA 5'
5' NONTRANSLATED REGION 10
START CODON
STRUCTURAL SEQUENCE 14
STOP CODON
3'NONTRANSLATED REGION 18
POLY-A "TAIL" 22
POLY-ADENYLATION SIGNAL 20
TRANSLATION BEGINS HERE
TRANSLATION ENDS HERE

FIG. 1.

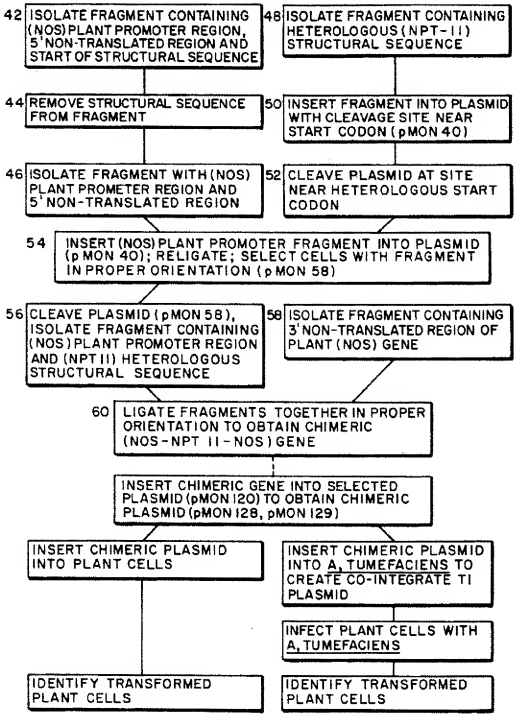

42
ISOLATE FRAGMENT CONTAINING (NOS) PLANT PROMOTER REGION, 5' NON-TRANSLATED REGION AND START OF STRUCTURAL SEQUENCE
48
ISOLATE FRAGMENT CONTAINING HETEROLOGOUS (NPT-II) STRUCTURAL SEQUENCE
44
REMOVE STRUCTURAL SEQUENCE FROM FRAGMENT
50
INSERT FRAGMENT INTO PLASMID WITH CLEAVAGE SITE NEAR START CODON (pMON 40)
46
ISOLATE FRAGMENT WITH (NOS) PLANT PROMETER REGION AND 5' NON-TRANSLATED REGION
52
CLEAVE PLASMID AT SITE NEAR HETEROLOGOUS START CODON
54
INSERT (NOS) PLANT PROMOTER FRAGMENT INTO PLASMID (p MON 40); RELIGATE; SELECT CELLS WITH FRAGMENT IN PROPER ORIENTATION (p MON 58)
56
CLEAVE PLASMID (pMON 58), ISOLATE FRAGMENT CONTAINING (NOS) PLANT PROMOTER REGION AND (NPT II) HETEROLOGOUS STRUCTURAL SEQUENCE
58
ISOLATE FRAGMENT CONTAINING 3' NON-TRANSLATED REGION OF PLANT (NOS) GENE
60
LIGATE FRAGMENTS TOGETHER IN PROPER ORIENTATION TO OBTAIN CHIMERIC (NOS-NPT II-NOS) GENE
INSERT CHIMERIC GENE INTO SELECTED PLASMID (pMON 120) TO OBTAIN CHIMERIC PLASMID (pMON 128, pMON 129)
INSERT CHIMERIC PLASMID INTO PLANT CELLS
INSERT CHIMERIC PLASMID INTO A. TUMEFACIENS TO CREATE CO-INTEGRATE TI PLASMID
INFECT PLANT CELLS WITH A. TUMEFACIENS
IDENTIFY TRANSFORMED PLANT CELLS
IDENTIFY TRANSFORMED PLANT CELLS

FIG. 2.

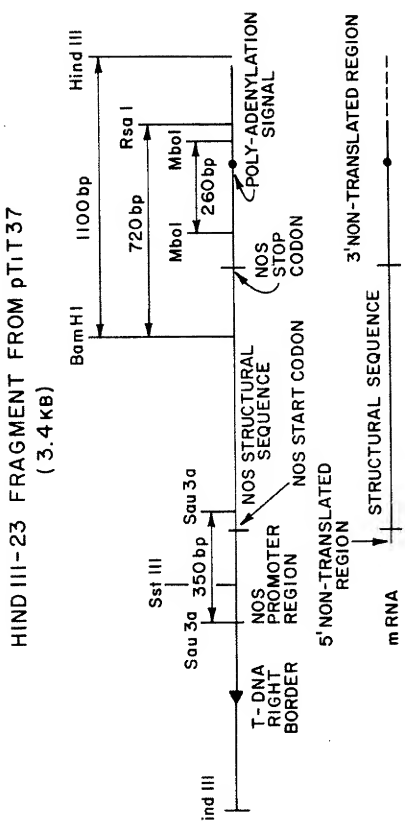

HIND III-23 FRAGMENT FROM pTiT37
(3.4 KB)
Hind III
T-DNA RIGHT BORDER
Sau 3a
350 bp
Sst III
Sau 3a
NOS PROMOTER REGION
NOS STRUCTURAL SEQUENCE
NOS START CODON
5' NON-TRANSLATED REGION
STRUCTURAL SEQUENCE
mRNA
BamHI
1100 bp
Hind III
720 bp
Rsa I
Mbol
Mbol
260 bp
NOS STOP CODON
POLY-ADENYLATION SIGNAL
3' NON-TRANSLATED REGION

FIG. 3.

Sau3a FRAGMENT CONTAINING
NOS PROMOTER REGION
(ANTI-SENSE STRAND, 344bp)

FIG. 4.

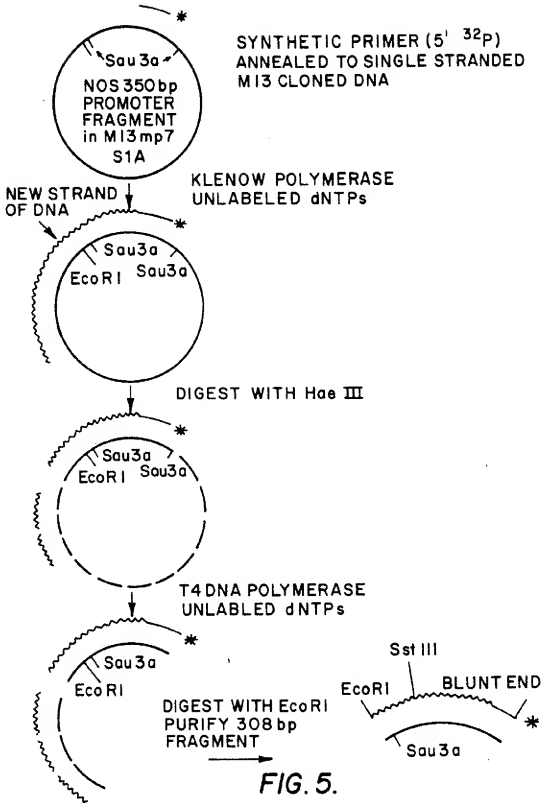

Sau 3a
NOS 350bp
PROMOTER
FRAGMENT
in M13mp7
S1A
SYNTHETIC PRIMER (5' 32P)
ANNEALED TO SINGLE STRANDED
M13 CLONED DNA
KLENOW POLYMERASE
UNLABELED dNTPs
NEW STRAND
OF DNA
Sau3a
EcoRI
Sau3a
DIGEST WITH Hae III
Sau3a
EcoRI
Sau3a
T4 DNA POLYMERASE
UNLABLED dNTPs
Sau3a
EcoRI
DIGEST WITH EcoRI
PURIFY 308 bp
FRAGMENT
Sst III
EcoRI
BLUNT END
Sau3a

FIG. 5.

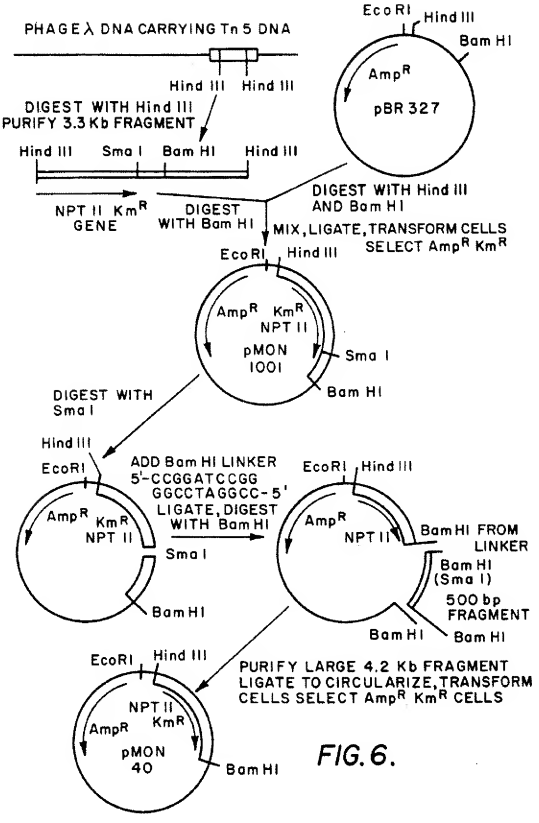

PHAGE λ DNA CARRYING Tn 5 DNA
Hind III
Hind III
DIGEST WITH Hind III
PURIFY 3.3 Kb FRAGMENT
Hind III
Sma I
Bam HI
Hind III
NPT II Km^R GENE
DIGEST WITH Bam HI
EcoRI
Hind III
Amp^R
pBR 327
DIGEST WITH Hind III AND Bam HI
MIX, LIGATE, TRANSFORM CELLS SELECT Amp^R Km^R
EcoRI
Hind III
Amp^R Km^R NPT II
pMON 1001
Sma I
Bam HI
DIGEST WITH Sma I
Hind III
EcoRI
ADD Bam HI LINKER
5'-CCGGATCCGG
GGCCTAGGCC-5'
LIGATE, DIGEST WITH Bam HI
Amp^R
Km^R
NPT II
Sma I
Bam HI
EcoRI
Hind III
Amp^R
NPT II
Bam HI FROM LINKER
Bam HI (Sma I)
500 bp FRAGMENT
Bam HI
Bam HI
EcoRI
Hind III
NPT II
Amp^R
Km^R
pMON 40
Bam HI
PURIFY LARGE 4.2 Kb FRAGMENT
LIGATE TO CIRCULARIZE, TRANSFORM
CELLS SELECT Amp^R Km^R CELLS

FIG. 6.

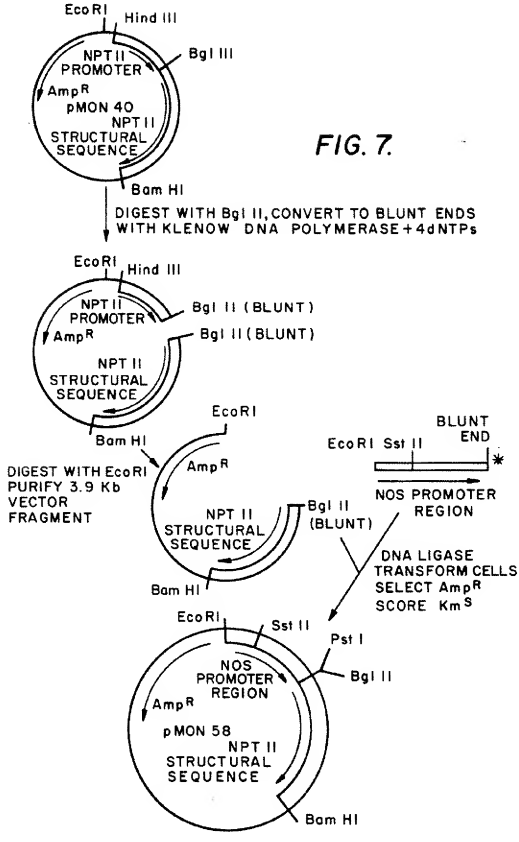

EcoRI
Hind III
Bgl III
NPT II PROMOTER
AmpR
pMON 40
NPT II STRUCTURAL SEQUENCE
Bam HI
DIGEST WITH Bgl II, CONVERT TO BLUNT ENDS WITH KLENOW DNA POLYMERASE + 4dNTPs
EcoRI
Hind III
NPT II PROMOTER
Bgl II (BLUNT)
AmpR
Bgl II (BLUNT)
NPT II STRUCTURAL SEQUENCE
Bam HI
EcoRI
DIGEST WITH EcoRI
PURIFY 3.9 Kb VECTOR FRAGMENT
AmpR
NPT II STRUCTURAL SEQUENCE
Bam HI
BLUNT END
EcoRI Sst II
NOS PROMOTER REGION
Bgl II (BLUNT)
DNA LIGASE
TRANSFORM CELLS
SELECT AmpR
SCORE KmS
EcoRI
Sst II
Pst I
Bgl II
NOS PROMOTER REGION
AmpR
pMON 58
NPT II STRUCTURAL SEQUENCE
Bam HI

FIG. 7.

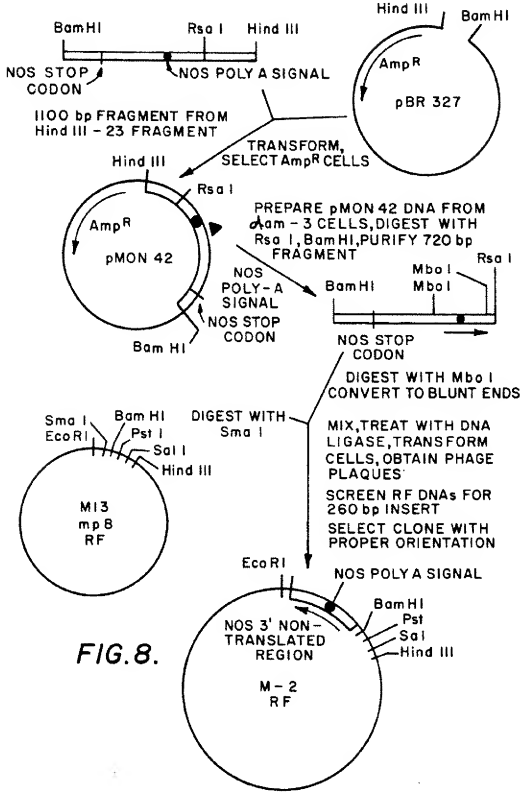

Bam HI
Rsa I
Hind III
NOS STOP CODON
NOS POLY A SIGNAL
1100 bp FRAGMENT FROM Hind III – 23 FRAGMENT
Hind III
Bam HI
Amp R
pBR 327
TRANSFORM, SELECT Amp R CELLS
Hind III
Rsa I
Amp R
pMON 42
Bam HI
PREPARE pMON 42 DNA FROM dam – 3 CELLS, DIGEST WITH Rsa I, Bam HI, PURIFY 720 bp FRAGMENT
NOS POLY-A SIGNAL
NOS STOP CODON
Bam HI
Mbo I
Mbo I
Rsa I
NOS STOP CODON
DIGEST WITH Mbo I CONVERT TO BLUNT ENDS
Sma I
Eco RI
Bam HI
Pst I
Sal I
Hind III
M13 mp 8 RF
DIGEST WITH Sma I
MIX, TREAT WITH DNA LIGASE, TRANSFORM CELLS, OBTAIN PHAGE PLAQUES
SCREEN RF DNAs FOR 260 bp INSERT
SELECT CLONE WITH PROPER ORIENTATION
Eco RI
NOS POLY A SIGNAL
Bam HI
Pst
Sal
Hind III
NOS 3' NON-TRANSLATED REGION
M-2 RF

FIG. 8.

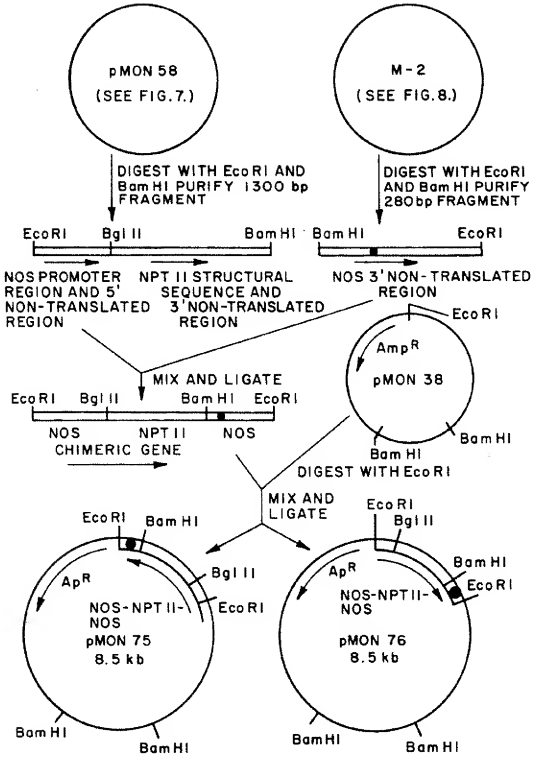

pMON 58
(SEE FIG. 7.)
M-2
(SEE FIG. 8.)
DIGEST WITH EcoRI AND BamHI PURIFY 1300 bp FRAGMENT
DIGEST WITH EcoRI AND BamHI PURIFY 280 bp FRAGMENT
EcoRI
BglII
BamHI
BamHI
EcoRI
NOS PROMOTER REGION AND 5' NON-TRANSLATED REGION
NPT II STRUCTURAL SEQUENCE AND 3'NON-TRANSLATED REGION
NOS 3'NON-TRANSLATED REGION
EcoRI
AmpR
pMON 38
BamHI
MIX AND LIGATE
EcoRI
BglII
BamHI
EcoRI
NOS
NPT II
NOS
CHIMERIC GENE
BamHI
DIGEST WITH EcoRI
MIX AND LIGATE
EcoRI
BamHI
EcoRI
BglII
ApR
BglII
EcoRI
NOS-NPT II-NOS
pMON 75
8.5 kb
ApR
BamHI
EcoRI
NOS-NPT II-NOS
pMON 76
8.5 kb
BamHI
BamHI
BamHI
BamHI

FIG. 9.

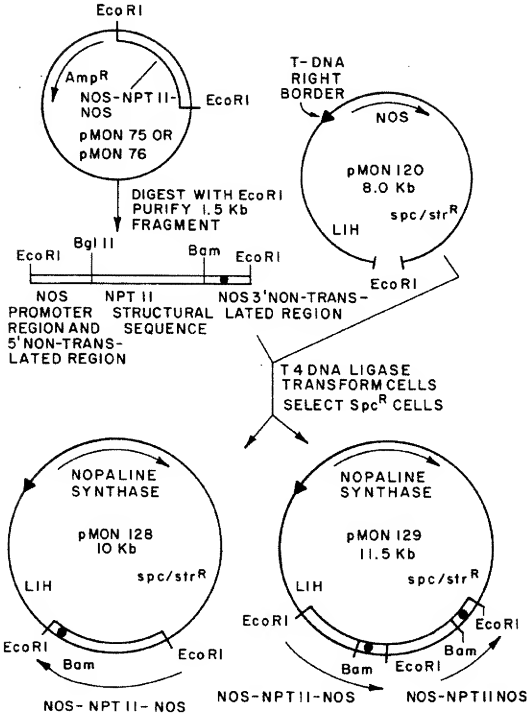

EcoRI
AmpR
NOS-NPT II-NOS
EcoRI
pMON 75 OR
pMON 76
DIGEST WITH EcoRI
PURIFY 1.5 Kb
FRAGMENT
T-DNA
RIGHT
BORDER
NOS
pMON 120
8.0 Kb
spc/str^R
LIH
EcoRI
EcoRI
Bgl II
Bam
EcoRI
NOS
PROMOTER
REGION AND
5'NON-TRANS-
LATED REGION
NPT II
STRUCTURAL
SEQUENCE
NOS 3'NON-TRANS-
LATED REGION
T4 DNA LIGASE
TRANSFORM CELLS
SELECT Spc^R CELLS
NOPALINE
SYNTHASE
pMON 128
10 Kb
spc/str^R
LIH
EcoRI
Bam
EcoRI
NOS-NPT II-NOS
NOPALINE
SYNTHASE
pMON 129
11.5 Kb
spc/str^R
LIH
EcoRI
EcoRI
Bam
Bam
EcoRI
NOS-NPT II-NOS
NOS-NPT II NOS

FIG. 10.

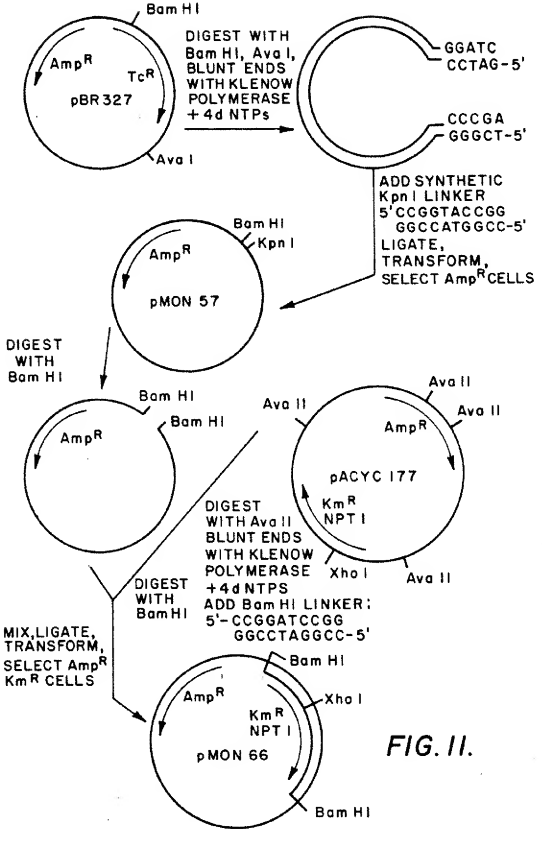

Bam HI
Amp^R
Tc^R
pBR 327
Ava I
DIGEST WITH Bam HI, Ava I, BLUNT ENDS WITH KLENOW POLYMERASE +4d NTPs
GGATC
CCTAG-5'
CCCGA
GGGCT-5'
ADD SYNTHETIC Kpn I LINKER 5'CCGGTACCGG GGCCATGGCC-5'
LIGATE, TRANSFORM, SELECT Amp^R CELLS
Bam HI
Kpn I
Amp^R
pMON 57
DIGEST WITH Bam HI
Bam HI
Bam HI
Amp^R
Ava II
Ava II
Ava II
Amp^R
pACYC 177
Km^R
NPT I
Xho I
DIGEST WITH Ava II BLUNT ENDS WITH KLENOW POLYMERASE +4d NTPS ADD Bam HI LINKER: 5'-CCGGATCCGG GGCCTAGGCC-5'
DIGEST WITH Bam HI
MIX, LIGATE, TRANSFORM, SELECT Amp^R Km^R CELLS
Bam HI
Amp^R
Xho I
Km^R
NPT I
pMON 66
Bam HI

FIG. 11.

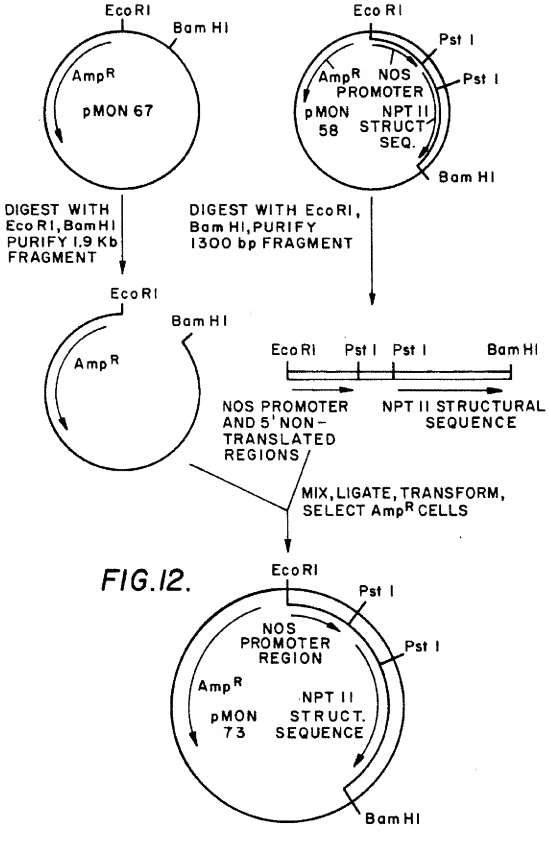

EcoRI
Bam HI
AmpR
pMON 67
EcoRI
Pst I
Pst I
AmpR
NOS PROMOTER
pMON 58
NPT II STRUCT SEQ.
Bam HI
DIGEST WITH EcoRI, BamHI PURIFY 1.9 Kb FRAGMENT
DIGEST WITH EcoRI, Bam HI, PURIFY 1300 bp FRAGMENT
EcoRI
Bam HI
AmpR
EcoRI
Pst I
Pst I
Bam HI
NOS PROMOTER AND 5' NON-TRANSLATED REGIONS
NPT II STRUCTURAL SEQUENCE
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
EcoRI
Pst I
NOS PROMOTER REGION
Pst I
AmpR
NPT II
pMON 73
STRUCT. SEQUENCE
Bam HI

FIG. 12.

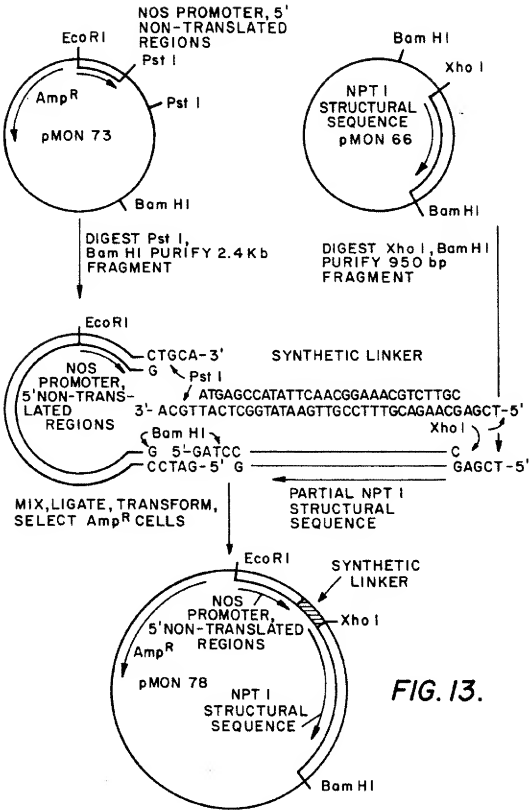

NOS PROMOTER, 5' NON-TRANSLATED REGIONS
EcoRI
Pst I
AmpR
Pst I
pMON 73
Bam HI
DIGEST Pst I, Bam HI PURIFY 2.4 Kb FRAGMENT
Bam HI
Xho I
NPT I STRUCTURAL SEQUENCE
pMON 66
Bam HI
DIGEST Xho I, Bam HI PURIFY 950 bp FRAGMENT
EcoRI
CTGCA-3'
SYNTHETIC LINKER
G
Pst I
NOS PROMOTER, 5'NON-TRANS-LATED REGIONS
ATGAGCCATATTCAACGGAAACGTCTTGC
3'- ACGTTACTCGGTATAAGTTGCCTTTGCAGAACGAGCT-5'
Bam HI
Xho I
G 5'-GATCC
C
CCTAG-5' G
GAGCT-5'
PARTIAL NPT I STRUCTURAL SEQUENCE
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
EcoRI
SYNTHETIC LINKER
NOS PROMOTER, 5'NON-TRANSLATED REGIONS
Xho I
AmpR
pMON 78
NPT I STRUCTURAL SEQUENCE
Bam HI

FIG. 13.

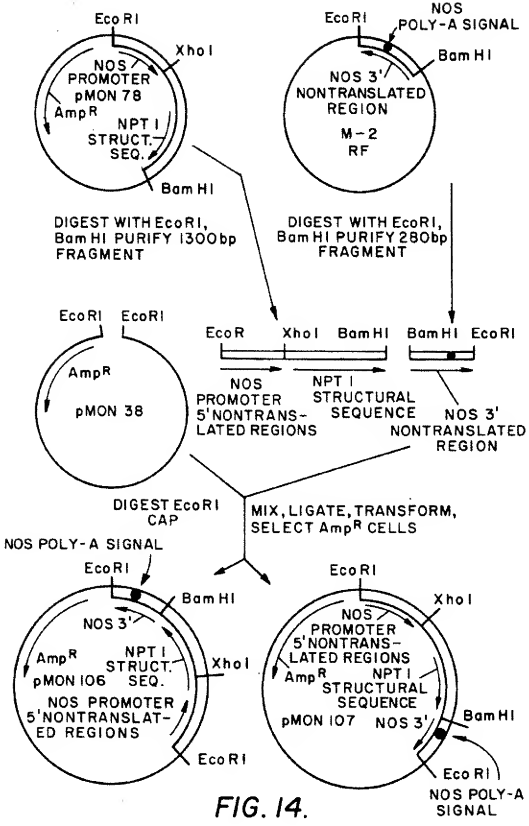

EcoRI
XhoI
NOS PROMOTER pMON 78
AmpR
NPT I STRUCT.-SEQ.
BamHI
DIGEST WITH EcoRI, BamHI PURIFY 1300bp FRAGMENT
EcoRI
NOS POLY-A SIGNAL
BamHI
NOS 3' NONTRANSLATED REGION
M-2 RF
DIGEST WITH EcoRI, BamHI PURIFY 280bp FRAGMENT
EcoRI EcoRI
AmpR
pMON 38
EcoR XhoI BamHI BamHI EcoRI
NOS PROMOTER 5'NONTRANS-LATED REGIONS
NPT I STRUCTURAL SEQUENCE
NOS 3' NONTRANSLATED REGION
DIGEST EcoRI CAP
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
NOS POLY-A SIGNAL
EcoRI
BamHI
NOS 3'
AmpR
NPT I STRUCT. SEQ.
pMON 106
XhoI
NOS PROMOTER 5'NONTRANSLAT-ED REGIONS
EcoRI
EcoRI
XhoI
NOS PROMOTER 5'NONTRANS-LATED REGIONS
AmpR
NPT I STRUCTURAL SEQUENCE
pMON 107
NOS 3'
BamHI
Eco RI
NOS POLY-A SIGNAL

FIG. 14.

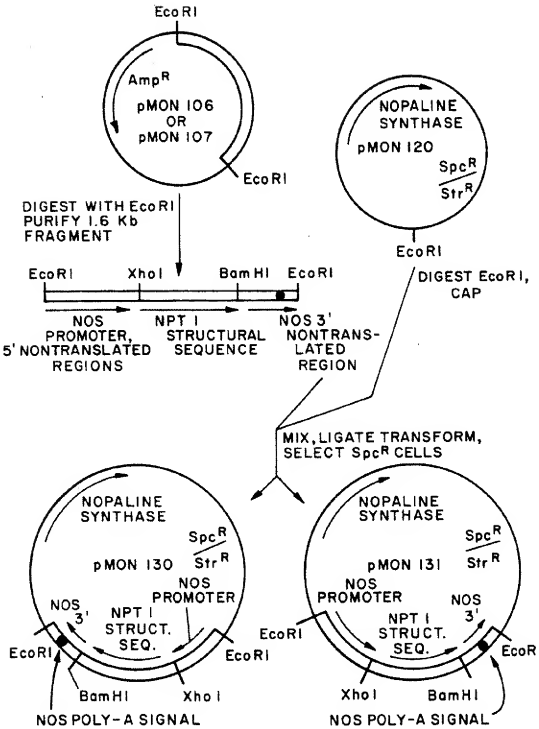

EcoRI
Amp^R
pMON 106
OR
pMON 107
EcoRI
DIGEST WITH EcoRI
PURIFY 1.6 Kb
FRAGMENT
NOPALINE
SYNTHASE
pMON 120
Spc^R
Str^R
EcoRI
DIGEST EcoRI,
CAP
EcoRI
XhoI
BamHI
EcoRI
NOS
PROMOTER,
5' NONTRANSLATED
REGIONS
NPT I
STRUCTURAL
SEQUENCE
NOS 3'
NONTRANS-
LATED
REGION
MIX, LIGATE TRANSFORM,
SELECT Spc^R CELLS
NOPALINE
SYNTHASE
Spc^R
Str^R
pMON 130
NOS
PROMOTER
NOS
3'
NPT I
STRUCT.
SEQ.
EcoRI
EcoRI
BamHI
XhoI
NOS POLY-A SIGNAL
NOPALINE
SYNTHASE
Spc^R
Str^R
pMON 131
NOS
PROMOTER
NOS
3'
NPT I
STRUCT.
SEQ.
EcoRI
EcoRI
XhoI
BamHI
NOS POLY-A SIGNAL

FIG. 15.

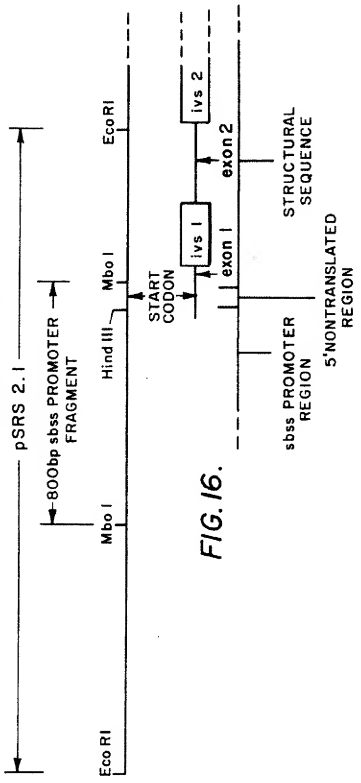

2.1 kb EcoRI FRAGMENT FROM
pSRS 2.1
800bp sbss PROMOTER FRAGMENT
Mbo I
Hind III
Mbo I
EcoRI
EcoRI
START CODON
ivs 1
ivs 2
exon 1
exon 2
STRUCTURAL SEQUENCE
sbss PROMOTER REGION
5'NONTRANSLATED REGION

FIG. 16.

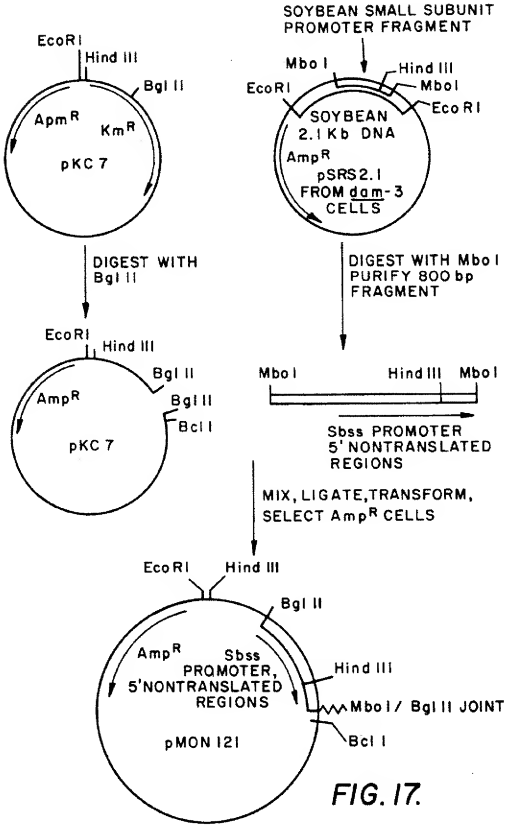
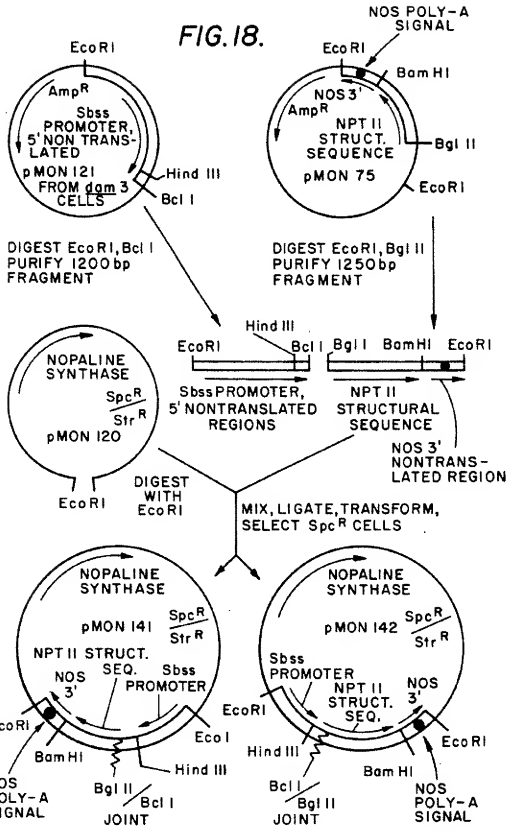

SOYBEAN SMALL SUBUNIT
PROMOTER FRAGMENT
EcoRI
Hind III
Bgl II
ApmR
KmR
pKC 7
Mbo I
Hind III
EcoRI
Mbo I
EcoRI
SOYBEAN
2.1 Kb DNA
AmpR
pSRS2.1
FROM dam-3
CELLS
DIGEST WITH
Bgl II
DIGEST WITH Mbo I
PURIFY 800 bp
FRAGMENT
EcoRI
Hind III
Bgl II
AmpR
Bgl II
Bcl I
pKC 7
Mbo I
Hind III
Mbo I
Sbss PROMOTER
5' NONTRANSLATED
REGIONS
MIX, LIGATE, TRANSFORM,
SELECT AmpR CELLS
EcoRI
Hind III
Bgl II
AmpR
Sbss
PROMOTER,
5'NONTRANSLATED
REGIONS
Hind III
Mbo I / Bgl II JOINT
Bcl I
pMON 121

FIG. 17.

FIG. 18.


NOS POLY-A SIGNAL
EcoRI
EcoRI
Bam HI
AmpR
Sbss PROMOTER, 5' NON TRANS-LATED
pMON 121 FROM dam 3 CELLS
Hind III
Bcl I
NOS 3'
AmpR
NPT II STRUCT. SEQUENCE
Bgl II
pMON 75
EcoRI
DIGEST EcoRI, Bcl I PURIFY 1200 bp FRAGMENT
DIGEST EcoRI, Bgl II PURIFY 1250 bp FRAGMENT
Hind III
EcoRI
Bcl I
Bgl I
BamHI
EcoRI
NOPALINE SYNTHASE
SpcR / StrR
pMON 120
Sbss PROMOTER, 5' NONTRANSLATED REGIONS
NPT II STRUCTURAL SEQUENCE
NOS 3' NONTRANS-LATED REGION
EcoRI
DIGEST WITH EcoRI
MIX, LIGATE, TRANSFORM, SELECT SpcR CELLS
NOPALINE SYNTHASE
pMON 141
SpcR / StrR
NPT II STRUCT. SEQ.
NOS 3'
Sbss PROMOTER
EcoRI
Bam HI
Hind III
Eco I
NOS POLY-A SIGNAL
Bgl II
Bcl I
JOINT
NOPALINE SYNTHASE
pMON 142
SpcR / StrR
Sbss PROMOTER
NPT II STRUCT. SEQ.
NOS 3'
EcoRI
Hind III
Bcl I
Bgl II
JOINT
Bam HI
EcoRI
NOS POLY-A SIGNAL

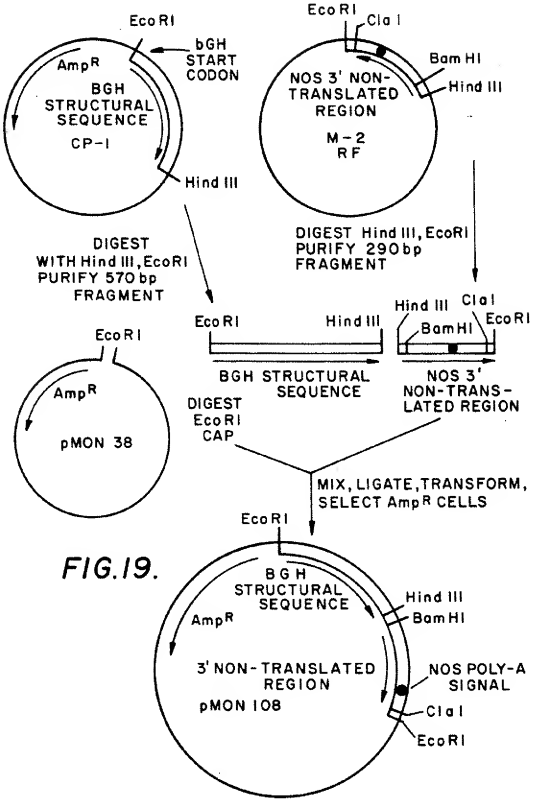

EcoRI
bGH START CODON
AmpR
BGH STRUCTURAL SEQUENCE
CP-I
Hind III
EcoRI
ClaI
BamHI
Hind III
NOS 3' NON-TRANSLATED REGION
M-2 RF
DIGEST WITH Hind III, EcoRI PURIFY 570 bp FRAGMENT
DIGEST Hind III, EcoRI PURIFY 290 bp FRAGMENT
EcoRI
AmpR
pMON 38
EcoRI
Hind III
BGH STRUCTURAL SEQUENCE
Hind III
ClaI
BamHI
EcoRI
NOS 3' NON-TRANS-LATED REGION
DIGEST EcoRI CAP
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
EcoRI
BGH STRUCTURAL SEQUENCE
AmpR
Hind III
BamHI
3' NON-TRANSLATED REGION
pMON 108
NOS POLY-A SIGNAL
ClaI
EcoRI

FIG.19.

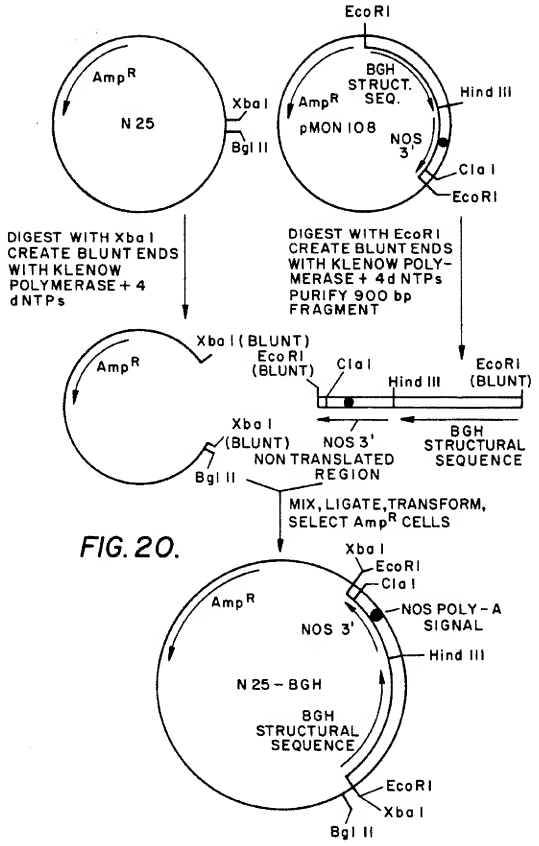

EcoRI
Amp^R
N 25
Xba I
Bgl II
BGH STRUCT. SEQ.
Amp^R
pMON 108
Hind III
NOS 3'
Cla I
EcoRI
DIGEST WITH Xba I CREATE BLUNT ENDS WITH KLENOW POLYMERASE + 4 dNTPs
DIGEST WITH EcoRI CREATE BLUNT ENDS WITH KLENOW POLY-MERASE + 4d NTPs PURIFY 900 bp FRAGMENT
Xba I (BLUNT)
EcoRI (BLUNT)
Amp^R
Cla I
Hind III
EcoRI (BLUNT)
Xba I (BLUNT)
Bgl II
NOS 3'
NON TRANSLATED REGION
BGH STRUCTURAL SEQUENCE
MIX, LIGATE, TRANSFORM, SELECT Amp^R CELLS
Xba I
EcoRI
Cla I
Amp^R
NOS POLY-A SIGNAL
NOS 3'
Hind III
N 25 – BGH
BGH STRUCTURAL SEQUENCE
EcoRI
Xba I
Bgl II

FIG. 20.

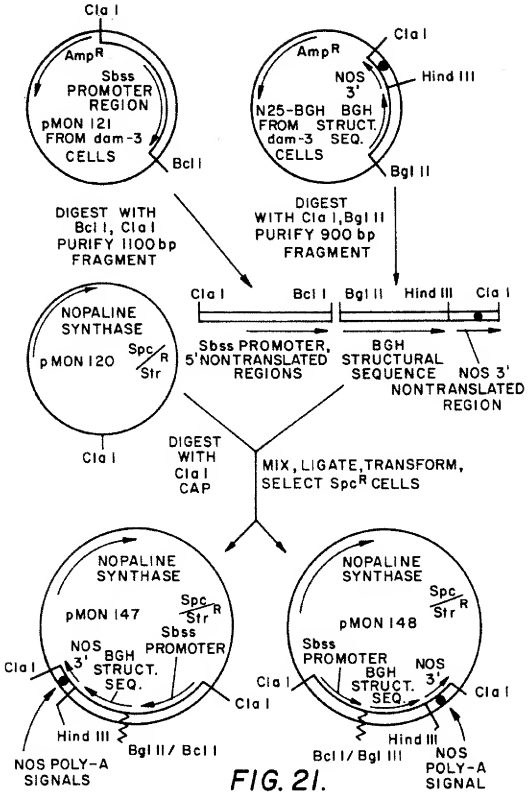

Cla I
AmpR
Sbss PROMOTER REGION
pMON 121 FROM dam-3 CELLS
Bcl I
Cla I
AmpR
NOS 3'
Hind III
N25-BGH FROM dam-3 CELLS
BGH STRUCT. SEQ.
Bgl II
DIGEST WITH Bcl I, Cla I PURIFY 1100 bp FRAGMENT
DIGEST WITH Cla I, Bgl II PURIFY 900 bp FRAGMENT
NOPALINE SYNTHASE
pMON 120
Spc/Str R
Cla I
Cla I
Bcl I
Bgl II
Hind III
Cla I
Sbss PROMOTER, 5'NONTRANSLATED REGIONS
BGH STRUCTURAL SEQUENCE
NOS 3' NONTRANSLATED REGION
DIGEST WITH Cla I CAP
MIX, LIGATE, TRANSFORM, SELECT SpcR CELLS
NOPALINE SYNTHASE
pMON 147
Spc/Str R
Sbss PROMOTER
NOS 3'
BGH STRUCT. SEQ.
Cla I
Cla I
Hind III
Bgl II/ Bcl I
NOS POLY-A SIGNALS
NOPALINE SYNTHASE
pMON 148
Spc/Str R
Sbss PROMOTER
BGH STRUCT. SEQ.
NOS 3'
Cla I
Cla I
Hind III
Bcl I/ Bgl III
NOS POLY-A SIGNAL

FIG. 21.

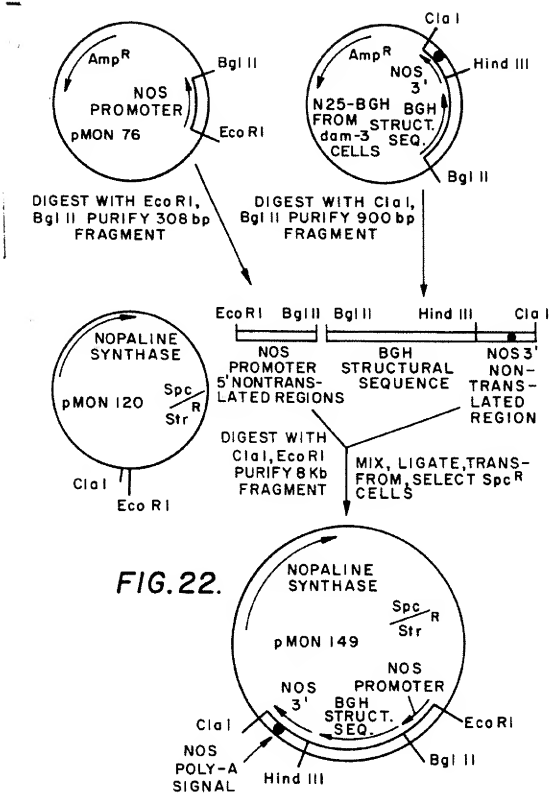

Amp R
Bgl II
NOS PROMOTER
pMON 76
EcoRI
Cla I
Amp R
Hind III
NOS 3'
N25-BGH FROM dam-3 CELLS
BGH STRUCT. SEQ.
Bgl II
DIGEST WITH EcoRI, Bgl II PURIFY 308 bp FRAGMENT
DIGEST WITH Cla I, Bgl II PURIFY 900 bp FRAGMENT
EcoRI Bgl II Bgl II Hind III Cla I
NOS PROMOTER 5' NONTRANS-LATED REGIONS
BGH STRUCTURAL SEQUENCE
NOS 3' NON-TRANS-LATED REGION
NOPALINE SYNTHASE
pMON 120
Spc R
Str
Cla I
Eco RI
DIGEST WITH Cla I, EcoRI PURIFY 8 Kb FRAGMENT
MIX, LIGATE, TRANS-FROM, SELECT Spc R CELLS
NOPALINE SYNTHASE
Spc R
Str
pMON 149
NOS PROMOTER
NOS 3'
BGH STRUCT. SEQ.
Cla I
EcoRI
NOS POLY-A SIGNAL
Hind III
Bgl II

FIG. 22.

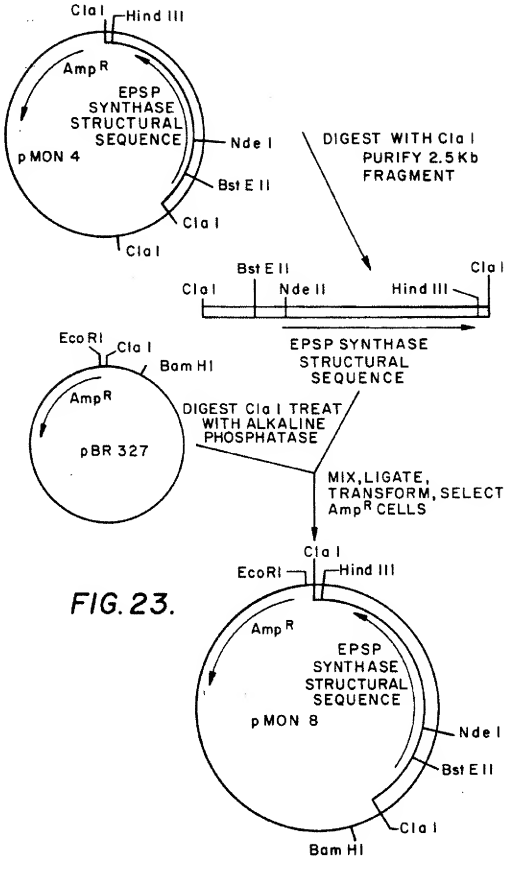

Cla I
Hind III
Amp R
EPSP SYNTHASE STRUCTURAL SEQUENCE
pMON 4
Nde I
Bst E II
Cla I
Cla I
DIGEST WITH Cla I
PURIFY 2.5 Kb FRAGMENT
Bst E II
Cla I
Cla I
Nde II
Hind III
EPSP SYNTHASE STRUCTURAL SEQUENCE
EcoRI
Cla I
Bam HI
Amp R
pBR 327
DIGEST Cla I TREAT WITH ALKALINE PHOSPHATASE
MIX, LIGATE, TRANSFORM, SELECT Amp R CELLS
Cla I
EcoRI
Hind III
Amp R
EPSP SYNTHASE STRUCTURAL SEQUENCE
pMON 8
Nde I
Bst E II
Cla I
Bam HI

FIG. 23.

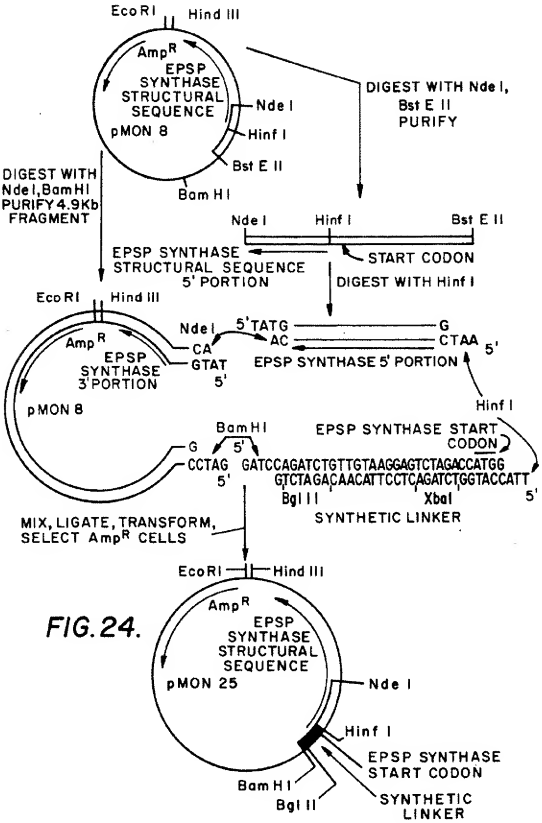

Eco RI
Hind III
Amp R
EPSP SYNTHASE STRUCTURAL SEQUENCE
pMON 8
Nde I
Hinf I
Bst E II
Bam H I
DIGEST WITH Nde I, Bst E II PURIFY
DIGEST WITH Nde I, Bam HI PURIFY 4.9Kb FRAGMENT
Nde I
Hinf I
Bst E II
EPSP SYNTHASE STRUCTURAL SEQUENCE 5' PORTION
START CODON
DIGEST WITH Hinf I
Eco RI
Hind III
Amp R
EPSP SYNTHASE 3' PORTION
pMON 8
Nde I
CA
GTAT
5'
5'TATG
AC
G
CTAA
5'
EPSP SYNTHASE 5' PORTION
Hinf I
Bam HI
5'
G
CCTAG
5'
EPSP SYNTHASE START CODON
GATCCAGATCTGTTGTAAGGAGTCTAGACCATGG
GTCTAGACAACATTCCTCAGATCTGGTACCATT
Bgl II
Xba I
5'
SYNTHETIC LINKER
MIX, LIGATE, TRANSFORM, SELECT Amp R CELLS
Eco RI
Hind III
Amp R
EPSP SYNTHASE STRUCTURAL SEQUENCE
pMON 25
Nde I
Hinf I
EPSP SYNTHASE START CODON
Bam HI
Bgl II
SYNTHETIC LINKER

FIG. 24.

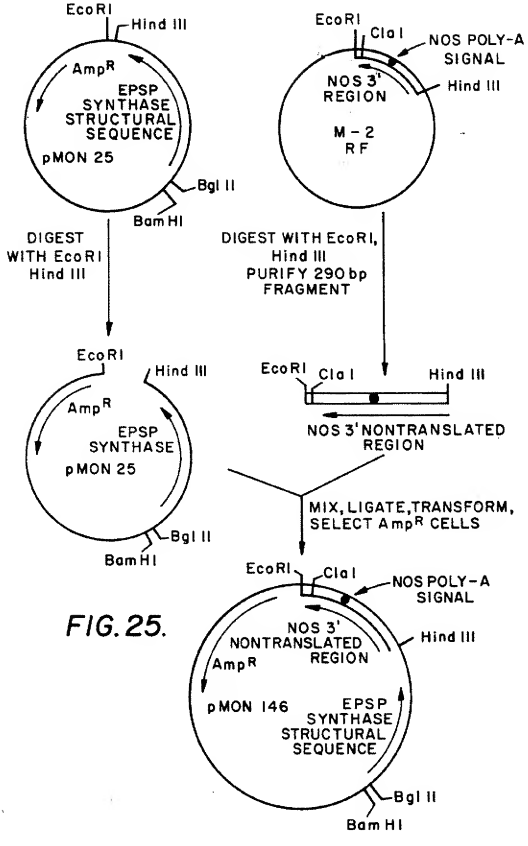

EcoRI
Hind III
AmpR
EPSP SYNTHASE STRUCTURAL SEQUENCE
pMON 25
Bgl II
Bam HI
DIGEST WITH EcoRI Hind III
EcoRI
Cla I
NOS POLY-A SIGNAL
NOS 3' REGION
Hind III
M-2 RF
DIGEST WITH EcoRI, Hind III PURIFY 290 bp FRAGMENT
EcoRI
Hind III
AmpR
EPSP SYNTHASE
pMON 25
Bgl II
Bam HI
EcoRI
Cla I
Hind III
NOS 3' NONTRANSLATED REGION
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
EcoRI
Cla I
NOS POLY-A SIGNAL
NOS 3' NONTRANSLATED REGION
Hind III
AmpR
pMON 146
EPSP SYNTHASE STRUCTURAL SEQUENCE
Bgl II
Bam HI

FIG. 25.

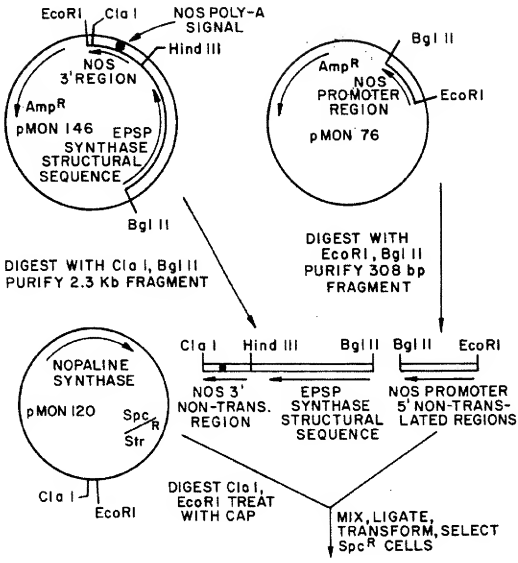
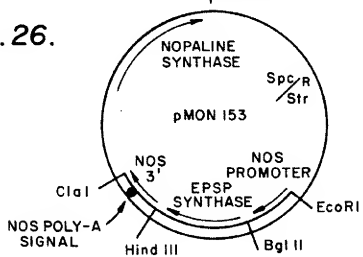

EcoRI
Cla I
NOS POLY-A SIGNAL
Hind III
NOS 3' REGION
AmpR
pMON 146
EPSP SYNTHASE STRUCTURAL SEQUENCE
Bgl II
Bgl II
AmpR
NOS PROMOTER REGION
EcoRI
pMON 76
DIGEST WITH Cla I, Bgl II PURIFY 2.3 Kb FRAGMENT
DIGEST WITH EcoRI, Bgl II PURIFY 308 bp FRAGMENT
NOPALINE SYNTHASE
pMON 120
Spc/Str R
Cla I
EcoRI
Cla I
Hind III
Bgl II
Bgl II
EcoRI
NOS 3' NON-TRANS. REGION
EPSP SYNTHASE STRUCTURAL SEQUENCE
NOS PROMOTER 5' NON-TRANSLATED REGIONS
DIGEST Cla I, EcoRI TREAT WITH CAP
MIX, LIGATE, TRANSFORM, SELECT SpcR CELLS

FIG. 26.


NOPALINE SYNTHASE
Spc/Str R
pMON 153
NOS 3'
NOS PROMOTER
EPSP SYNTHASE
Cla I
EcoRI
NOS POLY-A SIGNAL
Hind III
Bgl II

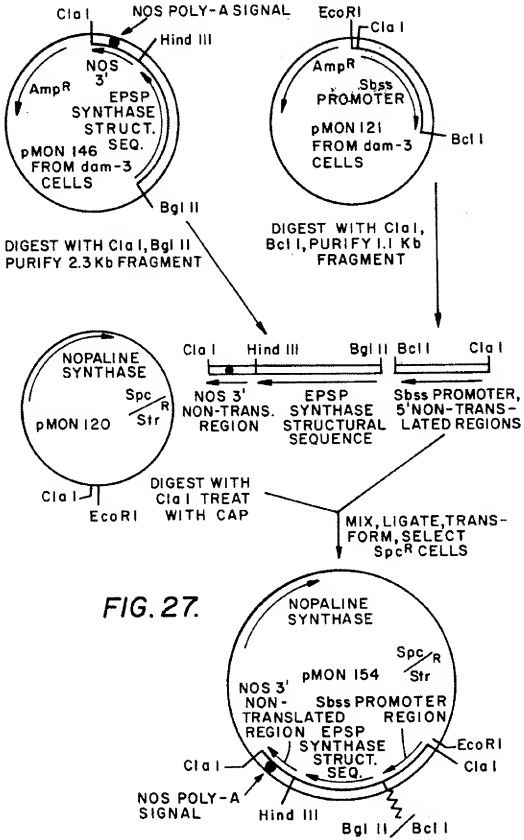

Cla I
NOS POLY-A SIGNAL
Hind III
NOS 3'
AmpR
EPSP SYNTHASE STRUCT. SEQ.
pMON 146 FROM dam-3 CELLS
Bgl II
DIGEST WITH Cla I, Bgl II PURIFY 2.3Kb FRAGMENT
EcoRI
Cla I
AmpR
Sbss PROMOTER
pMON 121 FROM dam-3 CELLS
Bcl I
DIGEST WITH Cla I, Bcl I, PURIFY 1.1 Kb FRAGMENT
NOPALINE SYNTHASE
Spc R
Str
pMON 120
Cla I
EcoRI
Cla I Hind III Bgl II Bcl I Cla I
NOS 3' NON-TRANS. REGION
EPSP SYNTHASE STRUCTURAL SEQUENCE
Sbss PROMOTER, 5'NON-TRANS-LATED REGIONS
DIGEST WITH Cla I TREAT WITH CAP
MIX, LIGATE, TRANS-FORM, SELECT SpcR CELLS
NOPALINE SYNTHASE
Spc R
Str
pMON 154
NOS 3' NON-TRANSLATED REGION
Sbss PROMOTER REGION
EPSP SYNTHASE STRUCT. SEQ.
EcoRI
Cla I
Cla I
NOS POLY-A SIGNAL
Hind III
Bgl II
Bcl I

FIG. 27.

CHIMERIC GENES SUITABLE FOR EXPRESSION IN PLANT CELLS

This is a continuation of application Ser. No. 06/793,488, filed Oct. 30, 1985, now abandoned, which is a continuation of application Ser. No. 06/458,414 filed Jan. 17, 1983, now abandoned.

TECHNICAL FIELD

This invention is in the fields of genetic engineering, plant biology, and bacteriology.

BACKGROUND ART

In the past decade, the science of genetic engineering has developed rapidly. A variety of processes are known for inserting a heterologous gene into bacteria, whereby the bacteria become capable of efficient expression of the inserted genes. Such processes normally involve the use of plasmids which may be cleaved at one or more selected cleavage sites by restriction endonucleases, discussed below. Typically, a gene of interest is obtained by cleaving one piece of DNA and the resulting DNA fragment is mixed with a fragment obtained by cleaving a vector such as a plasmid. The different strands of DNA are then connected ("ligated") to each other to form a reconstituted plasmid. See, for example, U.S. Pat. Nos. 4,237,224 (Cohen and Boyer, 1980); 4,264,731 (Shine, 1981); 4,273,875 (Manis, 1981); 4,322,499 (Baxter et al, 1982), and 4,336,336 (Silhavy et al, 1982). A variety of other reference works are also available. Some of these works describe the natural processes whereby DNA is transcribed into messenger (mRNA) and mRNA is translated into protein; see, e.g., Stryer, 1981 (note: all references cited herein, other than patents, are listed with citations after the Examples); Lehninger, 1975. Other works describe methods and products of genetic manipulation; see, e.g., Maniatis et al, 1982; Setlow and Hollaender, 1979.

Most of the genetic engineering work performed to date involves the insertion of genes into various types of cells primarily bacteria such as *E. coli*, various other types of microorganisms such as yeast, and mammalian cells. However, many of the techniques and substances used for genetic engineering of animal cells and microorganisms are not directly applicable to genetic engineering involving plants.

As used herein, the term "plant" refers to a multicellular differentiated organism that is capable of photosynthesis, such as angiosperms and multicellular algae. This does not include microorganisms, such as bacteria, yeast, and fungi. However, the term "plant cells" includes any cell derived from a plant; this includes undifferentiated tissue such as callus or crown gall tumor, as well as plant seeds, propagules, pollen, and plant embryos.

A variety of plant genes have been isolated, some of which have been published and/or are publicly available. Such genes include the soybean actin gene (Shah et al, 1982), corn zein (Pederson et al, 1982) soybean leghemoglobin (Hyldig-Nielsen et al, 1982), and soybean storage proteins (Fischer and Goldberg, 1982).

The Regions of a Gene

The expression of a gene involves the creation of a polypeptide which is coded for by the gene. This process involves at least two steps: part of the gene is transcribed to form messenger RNA, and part of the mRNA is translated into a polypeptide. Although the processes of transcription and translation are not fully understood, it is believed that the transcription of a DNA sequence into mRNA is controlled by several regions of DNA. Each region is a series of bases (i.e., a series of nucleotide residues comprising adenosine (A), thymidine (T), cytidine (C), and guanidine (G)) which are in a desired sequence. Regions which are usually present in a eucaryotic gene are shown on FIG. **1**. These regions have been assigned names for use herein, and are briefly discussed below. It should be noted that a variety of terms are used in the literature, which describes these regions in much more detail.

An association region **2** causes RNA polymerase to associate with the segment of DNA. Transcription does not occur at association region **2**; instead, the RNA polymerase normally travels along an intervening region **4** for an appropriate distance, such as about 100–300 bases, after it is activated by association region **2**.

A transcription initiation sequence **6** directs the RNA polymerase to begin synthesis of mRNA. After it recognizes the appropriate signal, the RNA polymerase is believed to begin the synthesis of mRNA an appropriate distance, such as about 20 to about 30 bases, beyond the transcription initiation sequence **6**. This is represented in FIG. **1** by intervening region **8**.

The foregoing sequences are referred to collectively as the promoter region of the gene.

The next sequence of DNA is transcribed by RNA polymerase into messenger RNA which is not translated into protein. In general, the 5' end of a strand of mRNA attaches to a ribosome. In bacterial cells, this attachment is facilitated by a sequence of bases called a "ribosome binding site" (RBS). However, in eucaryotic cells, no such RBS sequence is known to exist. Regardless of whether an RBS exists in a strand of mRNA, the mRNA moves through the ribosome until a "start codon" is encountered. The start codon is usually the series of three bases, AUG; rarely, the codon GUG may cause the initiation of translation. The non-translated portion of mRNA located between the 5' end of the mRNA and the start codon is referred to as the 5' non-translated region **10** of the mRNA. The corresponding sequence in the DNA is also referred to herein as 5' non-translated region **12**. The specific series of bases in this sequence is not believed to be of great importance to the expression of the gene; however, the presence of a premature start codon might affect the translation of the mRNA (see Kozak, 1978).

A promoter sequence may be significantly more complex than described above; for example, certain promoters present in bacteria contain regulatory sequences that are often referred to as "operators." Such complex promoters may contain one or more sequences which are involved in induction or repression of the gene. One example is the lac operon, which normally does not promote transcription of certain lactose-utilizing enzymes unless lactose is present in the cell. Another example is the trp operator, which does not promote transcription or translation of certain tryptophan-creating enzymes if an excess of tryptophan is present in the cell. See, e.g., Miller and Reznikoff, 1982.

The next sequence of bases is usually called the coding sequence or the structural sequence **14** (in the DNA molecule) or **16** (in the mRNA molecule). As mentioned above, the translation of a polypeptide begins when the mRNA start codon, usually AUG, reaches the transla-

tion mechanism in the ribosome. The start codon directs the ribosome to begin connecting a series of amino acids to each other by peptide bonds to form a polypeptide, starting with methionine, which always forms the amino terminal end of the polypeptide (the methionine residue may be subsequently removed from the polypeptide by other enzymes). The bases which follow the AUG start codon are divided into sets of 3, each of which is a codon. The "reading frame", which specifies how the bases are grouped together into sets of 3, is determined by the start codon. Each codon codes for the addition of a specific amino acid to the polypeptide being formed. The entire genetic code (there are 64 different codons) has been solved; see, e.g., Lehninger, supra, at p. 962. For example, CUA is the codon for the amino acid leucine; GGU specifies glycine, and UGU specifies cysteine.

Three of the codons (UAA, UAG, and UGA) are "stop" codons; when a stop codon reaches the translation mechanism of a ribosome, the polypeptide that was being formed disengages from the ribosome, and the last preceding amino acid residue becomes the carboxy terminal end of the polypeptide.

The region of mRNA which is located on the 3' side of a stop codon in a monocistronic gene is referred to herein as 3' non-translated region **18**. This region **18** is believed to be involved in the processing, stability, and/or transport of the mRNA after it is transcribed. This region **18** is also believed to contain a sequence of bases, poly-adenylation signal **20**, which is recognized by an enzyme in the cell. This enzyme adds a substantial number of adenosine residues to the mRNA molecule, to form poly-A tail **22**.

The DNA molecule has a 3' non-translated region **24** and a poly-adenylation signal **26**, which code for the corresponding mRNA region **18** and signal **20**. However, the DNA molecule does not have a poly-A tail. Poly-adenylation signals **20** (mRNA) and **26** (DNA) are represented in the figures by a heavy dot.

Gene-Host Incompatibility

The same genetic code is utilized by all living organisms on Earth. Plants, animals, and microorganisms all utilize the same correspondence between codons and amino acids. However, the genetic code applies only to the structural sequence of a gene, i.e., the segment of mRNA bounded by one start codon and one stop codon which codes for the translation of mRNA into polypeptides.

However, a gene which performs efficiently in one type of cell may not perform at all in a different type of cell. For example, a gene which is expressed in *E. coli* may be transferred into a different type of bacterial cell, a fungus, or a yeast. However, the gene might not be expressed in the new host cell. There are numerous reasons why an intact gene which is expressed in one type of cell might not be expressed in a different type of cell. See, e.g., Sakaguchi and Okanishi, 1981. Such reasons include:

1. the gene might not be replicated or stably inherited by the progeny of the new host cell.
2. the gene might be broken apart by restriction endonucleases or other enzymes in the new host cell.
3. the promoter region of the gene might not be recognized by the RNA polymerases in the new host cell.
4. one or more regions of the gene might be bound by a repressor protein or other molecule in the new host cell, because of a DNA region which resembles an operator or other regulatory sequence of the host's DNA. For example, the lac operon includes a polypeptide which binds to a particular sequence of bases next to the lac promoter unless the polypeptide is itself inactivated by lactose. See, e.g., Miller and Reznikoff, 1982.
5. one or more regions of the gene might be deleted, reorganized, or relocated to a different part of the host's genome. For example, numerous procaryotic cells are known to contain enzymes which promote genetic recombination (such as the rec proteins in *E. coli*; see, e.g., Shibata et al, 1979) and transposition (see, e.g., The 45th Cold Spring Harbor Symposium on Quantitative Biology, 1981). In addition, naturally-occurring genetic modification can be enhanced by regions of homology between different strands of DNA; see, e.g., Radding, 1978.
6. mRNA transcribed from the gene may suffer from a variety of problems. For example, it might be degraded before it reaches the ribosome, or it might not be poly-adenylated or transported to the ribosome, or it might not interact properly with the ribosome, or it might contain an essential sequence which is deleted by RNA processing enzymes.
7. the polypeptide which is created by translation of the mRNA coded for by the gene may suffer from a variety of problems. For example, the polypeptide may have a toxic effect on the cell, or it may be glycosylated or converted into an altered polypeptide, or it may be cleaved into shorter polypeptides or amino acids, or it may be sequestered within an intracellular compartment where it is not functional.

In general, the likelihood of a foreign gene being expressed in a cell tends to be lower if the new host cell is substantially different from the natural host cell. For example, a gene from a certain species of bacteria is likely to be expressed by other species of bacteria within the same genus. The gene is less likely to be expressed by bacteria of a different genus, and even less likely to be expressed by non-bacterial microorganisms such as yeast, fungus, or algae. It is very unlikely that a gene from a cell of one kingdom (the three kingdoms are plants, animals, and "protista" (microorganisms)) could be expressed in cells from either other kingdom.

These and other problems have, until now, thwarted efforts to obtain expression of foreign genes into plant cells. For example, several research teams have reported the insertion of foreign DNA into plant cells; see, e.g., Lurquin, 1979; Krens et al, 1982; Davey et al, 1980. At least three teams of researchers have reported the insertion of entire genes into plant cells. By use of radioactive DNA probes, these researchers have reported that the foreign genes (or at least portions thereof) were stably inherited by the descendants of the plant cells. See Hernalsteens et al, 1980; Garfinkel et al, 1981; Matzke and Chilton, 1981. However, there was no reported evidence that the foreign genes were expressed in the plant cells.

Several natural exceptions to the gene-host incompatibility barriers have been discovered. For example, several *E. coli* genes can be expressed in certain types of yeast cells, and vice-versa. See Beggs, 1978; Struhl et al, 1979.

In addition, certain types of bacterial cells, including *Agrobacterium tumefaciens* and *A. rhizogenes*, are capable of infecting various types of plant cells, causing plant diseases such as crown gall tumor and hairy root disease. These Agrobacterium cells carry plasmids,

designated as Ti plasmids and Ri plasmids, which carry genes which are expressed in plant cells. Certain of these genes code for enzymes which create substances called "opines," such as octopine, nopaline, and agropine. Opines are utilized by the bacteria cells as sources of carbon, nitrogen, and energy. See, e.g., Petit and Tempe, 1978. The opine genes are believed to be inactive while in the bacterial cells; these genes are expressed only after they enter the plant cells.

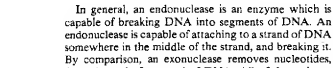
In addition, a variety of man-made efforts have been reported to overcome one or more of the gene-host incompatibility barriers. For example, it has been reported that a mammalian polypeptide which is normally degraded within a bacterial host can be protected from degradation by coupling the mammalian polypeptide to a bacterial polypeptide that normally exists in the host cell. This creates a "fusion protein;" see, e.g., Itakura et al, 1977. As another example, in order to avoid cleavage of an inserted gene by endonucleases in the host cell, it is possible to either (1) insert the gene into host cells which are deficient in one or more endonucleases, or (2) duplicate the gene in cells which cause the gene to be methylated. See, e.g., Maniatis et al, 1981.

In addition, various efforts to overcome gene-host incompatibility barriers involve chimeric genes. For example, a structural sequence which codes for a mammalian polypeptide, such as insulin, interferon, or growth hormone, may be coupled to regulatory sequences from a bacterial gene. The resulting chimeric gene may be inserted into bacterial cells, where it will express the mammalian polypeptide. See, e.g., Guarente et al, 1980. Alternately, structural sequences from several bacterial genes have been coupled to regulatory sequences from viruses which are capable of infecting mammalian cells. The resulting chimeric genes were inserted into mammalian cells, where they reportedly expressed the bacterial polypeptide. See, e.g., Southern and Berg, 1982; Colbere-Garapin et al, 1981.

Restriction Endonucleases

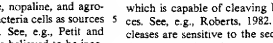
In general, an endonuclease is an enzyme which is capable of breaking DNA into segments of DNA. An endonuclease is capable of attaching to a strand of DNA somewhere in the middle of the strand, and breaking it. By comparison, an exonuclease removes nucleotides, from the end of a strand of DNA. All of the endonucleases discussed herein are capable of breaking double-stranded DNA into segments. This may require the breakage of two types of bonds: (1) covalent bonds between phosphate groups and deoxyribose residues, and (2) hydrogen bonds (A—T and C—G) which hold the two strands of DNA to each other.

A "restriction endonuclease" (hereafter referred to as an endonuclease) breaks a segment of DNA at a precise sequence of bases. For example, EcoRI and HaeIII recognize and cleave the following sequences:



In the examples cited above, the EcoRI cleavage created a "cohesive" end with a 5' overhang (i.e., the single-stranded "tail" has a 5' end rather than a 3' end). Cohesive ends can be useful in promoting desired ligations. For example, an EcoRI end is more likely to anneal to another EcoRI end than to a HaeIII end.

Over 100 different endonucleases are known, each of which is capable of cleaving DNA at specific sequences. See, e.g., Roberts, 1982. All restriction endonucleases are sensitive to the sequence of bases. In addition, some endonucleases are sensitive to whether certain bases have been methylated. For example, two endonucleases, MboI and Sau3a are capable of cleaving the following sequence of bases as shown:



MboI cannot cleave this sequence if the adenine residue is methylated (me-A). Sau3a can cleave this sequence, regardless of whether either A is methylated. To some extent the methylation (and therefore the cleavage) of a plasmid may be controlled by replicating the plasmids in cells with desired methylation capabilities. An *E. coli* enzyme, DNA adenine methylase (dam), methylates the A residues that occur in GATC sequences. Strains of *E. coli* which do not contain the dam enzyme are designated as dam⁻ cells. Cells which contain dam are designated as dam⁺ cells.

Several endonucleases are known which cleave different sequences, but which create cohesive ends which are fully compatible with cohesive ends created by other endonucleases. For example, at least five different endonucleases create 5' GATC overhangs, as shown in Table 1.

TABLE 1

Endonuclease	Sequence
MboI Inhibited by me-A	GATC CTAG
Sau3a Unaffected by me-A	same as MboI
BglII Unaffected by me-A	AGATCT TCTAGA
BclI Inhibited by me-A	TGATCA ACTAGT
BamHI Unaffected by me-A	GGATCC CCTAGG

A cohesive end created by any of the enconucleases listed in Table 1 will ligate preferentially to a cohesive end created by any of the other endonucleases. However, a ligation of, for example, a BglII end with a BamHI end will create the following sequence:

AGATCC

TCTAGG

This sequence cannot be cleaved by either Bgl II or BamHI; however, it can be cleaved by MboI (unless methylated) or by Sau3a.

Another endonuclease which involves the GATC sequence is PvuI, which creates a 3' overhang, as follows:

CGATCG
GCTAGC

Another endonuclease, ClaI, cleaves the following sequence:

X1ATCGATX2
Y TAGCTAY

If X_1 is G, or if X_2 is C, then the sequence may be cleaved by MboI (unless methylated, in which case ClaI is also inhibited) or Sau3a.

SUMMARY OF THE INVENTION

This invention relates to chimeric genes which are capable of being expressed in plant cells, and to a method for creating such genes.

The chimeric gene comprises a promoter region which is capable of causing RNA polymerase in a plant cell to create messenger RNA corresponding to the DNA. One such promoter region comprises a nopaline synthase (NOS) promoter region, which normally exists in certain types of Ti plasmids in bacteria, *A. tumefaciens*. The NOS gene normally is inactive while contained in *A. tumefaciens* cells, and it becomes active after the Ti plasmid enters a plant cell. Other suitable promoter regions may be derived from genes which exist naturally in plant cells.

The chimeric gene also contains a sequence of bases which codes for a 5' non-translated region of mRNA which is capable of enabling or increasing the expression in a plant cell of a structural sequence of the mRNA. For example, a suitable 5' non-translated region may be taken from the NOS gene mentioned above, or from a gene which exists naturally in plant cells.

The chimeric gene also contains a desired structural sequence, i.e., a sequence which is transcribed into mRNA which is capable of being translated into a desired polypeptide. The structural sequence is heterologous with respect to the promoter region, and it may code for any desired polypeptide, such as a bacterial or mammalian protein. The structural sequence includes a start codon and a stop codon. The structural sequence may contain introns which are removed from the mRNA prior to translation.

If desired, the chimeric gene may also contain a DNA sequence which codes for a 3' non-translated region (including a poly-adenylation signal) of mRNA. This region may be derived from a gene which is naturally expressed in plant cells, to help ensure proper expression of the structural sequence. Such genes include the NOS gene mentioned above, as well as genes which exist naturally in plant cells.

The method of this invention is described below, and is summarized in the flow chart of FIG. **2**.

If properly assembled and inserted into a plant genome, a chimeric gene of this invention will be expressed in the plant cell to create a desired polypeptide, such as a mammalian hormone, or a bacterial enzyme which confers antibiotic or herbicide resistance upon the plant.

BRIEF DESCRIPTION OF THE DRAWINGS

The figures herein are schematic representations; they have not been drawn to scale.

FIG. **1** represents the structure of a typical eukaryotic gene.

FIG. **2** is a flow chart representing the steps of this invention, correlated with an example chimeric NOS-NPTII-NOS gene

FIG. **3** represents fragment HindIII-23, obtained by digesting a Ti plasmid with HindIII.

FIG. **4** represents a DNA fragment which contains a NOS promoter region, a NOS 5' non-translated region, and the first few codons of the NOS structural sequence.

FIG. **5** represents the cleavage of a DNA sequence at a precise location, to obtain a DNA fragment which contains a NOS promoter region and complete 5' non-translated region.

FIG. **6** represents the creation of plasmids pMON1001 and pMON40, which contain an NPTII structural sequence.

FIG. **7** represents the insertion of a NOS promoter region into plasmid pMON40, to obtain pMON58.

FIG. **8** represents the creation of an M13 derivative designated as M-2, which contains a NOS 3' non-translated region and poly-A signal.

FIG. **9** represents the assembly of the NOS-NPTII-NOS chimeric gene, and the insertion of the chimeric gene into plasmid pMON38 to obtain plasmids pMON75 and pMON76.

FIG. **10** represents the insertion of the NOS-NPTII-NOS chimeric gene into plasmid pMON120 to obtain plasmids pMON128 and pMON129.

FIG. **11** represents the creation of plasmid pMON66, which contains an NPTI gene.

FIG. **12** represents the creation of plasmid pMON73, containing a chimeric NOS-NPTII sequence.

FIG. **13** represents the creation of plasmid pMON78, containing a chimeric NOS-NPTI sequence.

FIG. **14** represents the creation of plasmids pMON106 and pMON107, which contain chimeric NOS-NPTI-NOS genes.

FIG. **15** represents the insertion of a chimeric NOS-NPTI-NOS gene into pMON120 to obtain plasmids pMON130 and pMON131.

FIG. **16** represents the structure of a DNA fragment containing a soybean protein (sbss) promoter.

FIG. **17** represents the creation of plasmid pMON121, containing the sbss promoter.

FIG. **18** represents the insertion of a chimeric sbss-NPTII-NOS gene into pMON120 to create plasmids pMON141 and pMON142.

FIG. **19** represents the creation of plasmid pMON108, containing a bovine growth hormone structural sequence and a NOS 3' region.

FIG. **20** represents the creation of plasmid N25-BGH, which contains the BGH-NOS sequence surrounded by selected cleavage sites.

FIG. **21** represents the insertion of a chimeric sbss-BGH-NOS gene into pMON120 to obtain plasmids pMON147 and pMON148.

FIG. **22** represents the creation of plasmid pMON149, which contains a chimeric NOS-BGH-NOS gene.

FIG. **23** represents the creation of plasmid pMON8, which contains a structural sequence for EPSP synthase.

FIG. **24** represents the creation of plasmid pMON25, which contains an EPSP synthase structural sequence with several cleavage site near the start codon.

FIG. **25** represents the creation of plasmid pMON146, which contains a chimeric sequence comprising EPSP synthase and a NOS 3' region.

FIG. **26** represents the insertion of a chimeric NOS-EPSP-NOS gene into pMON120 to obtain plasmid pMON153.

FIG. **27** represents the creation of plasmid pMON154, which contains a chimeric sbss-EPSP-NOS gene.

DETAILED DESCRIPTION OF THE INVENTION

In one preferred embodiment of this invention, a chimeric gene was created which contained the following elements:

1. a promoter region and a 5' non-translated region derived from a nopaline synthase (NOS) gene;
2. a structural sequence derived from a neomycin phosphotransferase II (NPTII) gene; and,
3. a 3' non-translated region, including a poly-adenylation signal, derived from a NOS gene.

This chimeric gene, referred to herein as a NOS-NPTII-NOS gene, was assembled and inserted into a variety of plant cells, causing them to become resistant to aminoglycoside antibiotics such as kanamycin.

The method used to assemble this chimeric gene is summarized in the flow chart of FIG. **2**, and described in detail below and in the examples. To assist the reader in understanding the steps of this method, various plasmids and fragments involved in the NOS-NPTII-NOS chimeric gene are cited in parentheses in FIG. **2**. However, the method of FIG. **2** is applicable to a wide variety of other plasmids and fragments. To further assist the reader, the steps shown in FIG. **2** have been assigned callout numbers 42 et seq. These callout numbers are cited in the following description. The techniques and DNA sequences of this invention are likely to be useful in the transformation of a wide variety of plants, including any plant which may be infected by one or more strains of *A. tumefaciens* or *A. rhizogenes*.

The NOS Promoter Region and 5' Non-translated Region

The Applicants decided to obtain and utilize a nopaline synthase (NOS) promoter region to control the expression of the heterologous gene. The NOS is normally carried in certain types of Ti plasmids, such as pTiT37. Sciaky et al, 1978. The NOS promoter is normally inactive while in an *A. tumefaciens* cell. The entire NOS gene, including the promoter and the protein coding sequence, is within the T-DNA portion of a Ti plasmid that is inserted into the chromosomes of plant cells when a plant becomes infected and forms a crown gall tumor. Once inside the plant cell, the NOS promoter region directs RNA polymerase within a plant cell to transcribe the NOS protein coding sequence into mRNA, which is subsequently translated into the NOS enzyme.

The boundaries between the different parts of a promoter region (shown in FIG. **1** as association region **2**, intervening region **4**, transcription initiation sequence **6**, and intervening region **8**), and the boundary between the promoter region and the 5' non-translated region, are not fully understood. The Applicants decided to utilize the entire promoter region and 5' non-translated region from the NOS gene, which is known to be expressed in plant cells. However, it is entirely possible that one or more of these sequences might be modified in various ways, such as alteration in length or replacement by other sequences. Such modifications in promoter regions and 5' non-translated regions have been studied in bacterial cells (see, e.g., Roberts et al 1979) and mammalian cells (see, e.g., McKnight, 1982). By utilizing the methodology taught by this invention, it is now possible to study the effects of modifications to promoter regions and 5' non-translated regions on the expression of genes in plant cells. It may be possible to increase the expression of a gene in a plant cell by means of such modifications. Such modifications, if performed upon chimeric genes of this invention, are within the scope of this invention.

A nopaline-type tumor-inducing plasmid, designated as pTiT37, was isolated from a strain of *A. tumefaciens* using standard procedures (Currier and Nester, 1976). It was digested with the endonuclease HindIII which produced numerous fragments. These fragments were separated by size on a gel, and one of the fragments was isolated and removed from the gel. This fragment was designated as the HindIII-23 fragment, because it was approximately the 23rd largest fragment from the Ti plasmid; it is approximately 3400 base pairs (bp) in size, also referred to as 3.4 kilobases (kb). From work by others (see, e.g., Hernalsteens et al, 1980), it was known that the HindIII-23 fragment contained the entire NOS gene, including the promoter region, a 5' non-translated region, a structural sequence with a start codon and a stop codon, and a 3' non-translated region. The HindIII-23 fragment is shown in FIG. **3**.

By means of various cleavage and sequencing experiments, it was determined that the HindIII-23 fragment could be digested by another endonuclease, Sau3a, to yield a fragment, about 350 bp in size, which contains the entire NOS promoter region, the 5' non-translated region, and the first few codons of the NOS structural sequence. This fragment was sequenced, and the base sequence is represented in FIG. **4**. The start codon (ATG) of the NOS structural sequence begins at base pair **301** within the 350 bp fragment. The Applicants decided to cleave the fragment between base pairs **300** and **301**; this would provide them with a fragment about 300 base pairs long containing a NOS promoter region and the entire 5' non-translated region but with no translated bases. To cleave the 350 bp fragment at precisely the right location, the Applicants obtained an M13 clone designated as SIA, and utilized the procedure described below.

To create the SIA clone, Dr. Michael Bevan of Washington University converted the 350 bp Sau3a fragment into a single strand of DNA. This was done by utilizing a virus vector, designated as the M13 mp2 phage, which goes through both double-stranded (ds) and single-stranded (ss) stages in its life cycle (Messing et al, 1981). The ds 350 bp fragment was inserted into the double-stranded replicative form DNA of the M13 mp2, which had been cleaved with BamHI. The two fragments were ligated, and used to infect *E. coli* cells. The ds DNA containing the 350 bp inserted fragment subsequently replicated, and one strand (the viral strand) was encapsulated by the M13 viral capsid proteins. In one clone, designated the SIA, the orientation of the 350 bp fragment was such that the anti-sense strand (containing the same sequence as the mRNA) of the NOS gene was carried in the viral strand. Viral particles released from infected cells were isolated, and provided to the Applicants.

Single stranded SIA DNA, containing the anti-sense 350 bp fragment with the NOS promoter region, was isolated from the viral particles and sequenced. A 14-mer oligonucleotide primer was synthesized, using published procedures (Beaucage and Carruthers, 1981, as modified by Adams et al, 1982). This 14-mer was designed to be complementary to bases 287 through 300 of the 350 bp fragment, as shown on FIG. 4.

The 5' end of the synthetic primer was radioactively labelled with ^{32}P; this is represented in the figures by an asterisk

Copies of the primer were mixed with copies of the single-stranded SIA DNA containing the anti-sense strand of the 350 bp fragment. The primer annealed to the desired region of the SIA DNA, as shown at the top of FIG. 5. After this occurred, Klenow DNA polymerase and a controlled quantity of unlabelled deoxynucleoside triphosphates (dNTP's), A, T, C, and G, were added. Klenow polymerase added nucleotides to the 3' (unlabelled) end of the primer, but not to the 5' (labelled) end. The result, as shown in FIG. 5, was a circular loop of single-stranded DNA, part of which was matched by a second strand of DNA. The 5' end of the second strand was located opposite base #300 of the Sau3a insert

The partially double-stranded DNA was then digested by a third endonuclease, HaeIII, which can cleave both single-stranded and double-stranded DNA. HaeIII cleavage sites were known to exist in several locations outside the 350 bp insert, but none existed inside the 350 bp insert. This created a fragment having one blunt end, and one 3' overhang which started at base #301 of the Sau3a insert.

The HaeIII fragment mixture was treated with T4 DNA polymerase and unlabelled dNTP's. This caused the single stranded portion of the DNA, which extended from base #301 of the Sau3a insert to the closest HaeIII cleavage site, to be removed from the fragment. In this manner, the ATG start codon was removed from base pair #300, leaving a blunt end double-stranded fragment which was approximately 550 bp long.

The mixture was then digested by a fourth endonuclease EcoRI, which cleaved the 550 bp fragment at a single site outside the NOS promoter region. The fragments were then separated by size on a gel, and the radioactively-labelled fragment was isolated. This fragment contained the entire NOS promoter region and 5' non-translated region. It had one blunt end with a sequence of

```
5'- . . . CTGCA
    . . . GACGT
```

and one cohesive end (at the EcoRI site) with a sequence of

```
5' AATTC—
      G—
```

The shorter strand was about 308 bp long.

The foregoing steps are represented in FIG. 2 as steps 42, 44, and 46.

This fragment was inserted into pMON40 (which is described below) to obtain pMON58, as shown on FIG. 7.

Creation of plasmid with NPT II gene (pMON40)

A bacterial transposon, designated as Tn5, is known to contain a complete NPT II gene, including promoter region, structural sequence, and 3' non-translated region. The NPT II enzyme inactivates certain aminoglycoside antibiotics, such as kanamycin, neomycin, and G418; see Jimenez and Davies, 1980. This gene is contained within a 1.8 kb fragment, which can be obtained by digesting phage lambda bbkan-1 DNA (D. Berg et al, 1975) with two endonucleases, HindIII and BamHI. This fragment was inserted into a common laboratory plasmid, pBR327, which had been digested by HindIII and BamHI. As shown in FIG. 6, the resulting plasmid was designated as pMON1001, which was about 4.7 kb.

To reduce the size of the DNA fragment which carried the NPT II structural sequence, the Applicants eliminated about 500 bp from the pMON1001 plasmid, in the following manner. First, they digested pMON1001 at a unique SmaI restriction site which was outside of the NPT II gene. Next, they inserted a 10-mer synthetic oligonucleotide linker,

```
5' CCGGATCCGG
     GGCCTAGGCC
```

into the SmaI cleavage site. This eliminated the SmaI cleavage site and replaced it with a BamHI cleavage site. A second BamHI cleavage site already existed, about 500 bp from the new BamHI site. The Applicants digested the plasmid with BamHI, separated the 500 bp fragment from the 4.2 kb fragment, and circularized the 4.2 kb fragment. The resulting plasmids were inserted into *E. coli*, which were then selected for resistance to ampicillin and kanamycin. A clonal colony of *E. coli* was selected; ;these cells contained a plasmid which was designated as pMON40, as shown in FIG. 6.

The foregoing steps are represented in FIG. 2 as steps 48 and 50.

Insertion of NOS promoter into plasmid pMON40

The Applicants deleted the NPT II promoter from pMON40, and replaced it with the NOS promoter fragment described previously, by the following method, shown on FIG. 7.

Previous cleavage and sequencing experiments (Rao and Rogers, 1979; Auerswald et al, 1980) indicated that a BglII cleavage site existed in the NPT II gene between the promoter region and the structural sequence. Plasmid pMON40 was digested with BglII. The cohesive ends were then filled in by mixing the cleaved plasmid with Klenow polymerase and the four dNTP's, to obtain the following blunt ends:

```
5' —AGATC    GATCT—
   —TCTAG    CTAGA-5'
```

The polymerase and dNTP's were removed, and the cleaved plasmid was then digested with EcoRI. The smaller fragment which contained the NPT II promoter region was removed, leaving a large fragment with one EcoRI end and one blunt end. This large-fragment was mixed with the 308 bp fragment which contained the NOS promoter, described previously and shown on FIG. 5. The fragments were ligated, and inserted into *E. coli*. *E. coli* clones were selected for ampicillin resis-

tance. Replacement of the NPT II promoter region (a bacterial promoter) with the NOS promoter region (which is believed to be active only in plant cells) caused the NPT II structural sequence to become inactive in *E. coli*. Plasmids from 36 kanamycin-sensitive clones were obtained; the plasmid from one clone, designated as pMON58, was utilized in subsequent work.

The foregoing steps are represented in FIG. 2 as steps 52 and 54.

Plasmid pMON58 may be digested to obtain a 1.3 kb EcoRI-BamHI fragment which contains the NOS promoter region, the NOS 5' non-translated region, and the NPT II structural sequence. This step is represented in FIG. 2 as step 56.

Insertion of NOS 3' sequence into NPT II gene

As mentioned above in "Background Art", the functions of 3' non-translated regions in eucaryotic genes are not fully understood. However, they are believed to contain at least one important sequence, a poly-adenylation signal.

It was suspected by the Applicants that a gene having a bacterial 3' non-translated region might not be expressed as effectively in a plant cell as the same gene having a 3' non-translated region from a gene, such as NOS, which is known to be expressed in plants. Therefore, the Applicants decided to add a NOS 3' non-translated region to the chimeric gene, in addition to the NPT II 3' non-translated region already present. Whether a different type of 3' non-translated region (such as a 3' region from an octopine-type or agropine-type Ti plasmid, or a 3' region from a gene that normally exists in a plant cell) would be suitable or preferable for use in any particular type of chimeric gene, for use in any specific type of plant cell, may be determined by those skilled in the art through routine experimentation using the method of this invention. Alternately, it is possible, using the methods described herein, to delete the NPT II or other existing 3' non-translated region and replace it with a desired 3' non-translated region that is known to be expressed in plant cells.

Those skilled in the art may also determine through routine experimentation whether the 3' non-translated region that naturally follows a structural sequence that is to be inserted into a plant cell will enhance the efficient expression of that structural sequence in that type of plant cell. If so, then the steps required to insert a different 3' non-translated region into the chimeric gene might not be required in order to perform the method of this invention.

In order to obtain a DNA fragment containing a NOS 3' non-translated region appropriate for joining to the NPT II structural sequence from pMON58 (described previously), the Applicants utilized a 3.4 kb HindIII-23 fragment from a Ti plasmid, shown on FIG. 3. This 3.4 kb fragment was isolated and digested with BamHI to obtain a 1.1 kb BamHI-HindIII fragment containing a 3' portion of the NOS structural sequence (including the stop codon), and the 3' non-translated region of the NOS gene (including the poly-adenylation signal). This 1.1 kb fragment was inserted into a pBR327 plasmid which had been digested with HindIII and BamHI. The resulting plasmid was designated as pMON42, as shown on FIG. 8.

Plasmid pMON42 was digested with BamHI and RsaI, and a 720 bp fragment containing the desired NOS 3' non-translated region was purified on a gel. The 720 bp fragment was digested with another endonuclease, MboI, and treated with the large fragment of *E. coli* DNA polymerase I. This resulted in a 260 bp fragment with MboI blunt ends, containing a large part of the NOS 3' non-translated region including the poly-A signal.

The foregoing procedure is represented in FIG. 2 by step 58. However, it is recognized that alternate means could have been utilized; for example, it might have been possible to digest the HindIII-23 fragment directly with MboI to obtain the desired 260 bp fragment with the NOS 3' non-translated region.

Assembly of Chimeric Gene

To complete the assembly of the chimeric gene, it was necessary to ligate the 260 bp MboI fragment (which contained the NOS 3' non-translated region) to the 1.3 kb EcoRI-BamHI fragment from pMON58 (which contained the NOS promoter region and 5' non-translated region and the NPT II structural sequence). In order to facilitate this ligation and control the orientation of the fragments, the Applicants decided to convert the MboI ends of the 260 bp fragment into a BamHI end (at the 5' end of the fragment) and an EcoRI end (at the 3' end of the fragment). In order to perform this step, the Applicants used the following method.

The 260 bp MboI fragment, the termini of which had been converted to blunt ends by Klenow polymerase, was inserted into M13 mp8 DNA at a SmaI cleavage site. The SmaI site is surrounded by a variety of other cleavage sites present in the M13 mp8 DNA, as shown in FIG. 8. The MboI fragment could be inserted into the blunt SmaI ends in either orientation. The orientation of the MboI fragments in different clones were tested, using HinfI cleavage sites located assymetrically within the MboI fragment. A clone was selected in which the 3' end of the NOS 3' non-translated region was located near the EcoRI cleavage site in the M13 mp8 DNA. This clone was designated as the M-2 clone, as shown in FIG. 8.

Replicative form (double stranded) DNA from the M-2 clone was digested by EcoRI and BamHI and a 280 bp fragment was isolated. Separately, plasmid pMON58 was digested by EcoRI and BamHI, and a 1300 bp fragment was isolated. The two fragments were ligated, as shown in FIG. 9, to complete the assembly of a NOS-NPTII-NOS chimeric gene having EcoRI ends.

There are a variety of ways to control the ligation of the two fragments. For example, the two EcoRI-BamHI fragments could be joined together with DNA ligase and cleaved with EcoRI. After inactivation of EcoRI, a vector molecule having EcoRI ends that were treated with calf alkaline phosphatase (CAP) may be added to the mixture. The fragments in the mixture may be ligated in a variety of orientations. The plasmid mixture is used to transform *E. coli*, and cells having plasmids with the desired orientation are selected or screened, as described below.

A plasmid, designated as pMON38, was created by insertion of the HindIII-23 fragment (from Ti plasmid pTiT37) into the HindIII cleavage site of the plasmid pBR327. Plasmid pMON38 contains a unique EcoRI site, and an ampicillin-resistance gene which is expressed in *E. coli*. Plasmid pMON38 was cleaved with EcoRI and treated with alkaline phosphatase to prevent it from re-ligating to itself. U.S. Pat. No. 4,264,731 (Shine, 1981). The resulting fragment was mixed with the 1300 bp NOS-NPTII fragment from pMON58, and

the 280 bp NOS fragment from M-2, which had been ligated and EcoRI-cleaved as described in the previous paragraph. The fragments were ligated, and inserted into *E. coli.* The *E. coli* cells which had acquired intact plasmids with ampicillin-resistance genes were selected on plates containing ampicillin. Several clones were selected, and the orientation of the inserted chimeric genes was evaluated by means of cleavage experiments. Two clones having plasmids carrying NOS-NPT II-NOS inserts with opposite orientations were selected and designated as pMON75 and pMON76, as shown in FIG. **9**. The chimeric gene may be isolated by digesting either pMON75 or pMON76 with EcoRI and purifying a 1580 bp fragment.

The foregoing procedure is represented on FIG. **2** by step 60.

This completes the discussion of the NOS-NPTII-NOS chimeric gene. Additional information on the creation of this gene is provided in the Examples. A copy of this chimeric gene is contained in plasmid pMON128; it may be removed from pMON128 by digestion with EcoRI. A culture of *E. coli* containing pMON128 has been deposited with the American Type Culture Collection; this culture has been assigned accession number 39264.

To prove the utility of this chimeric gene, the Applicants inserted it into plant cells. The NPTII structural sequence was expressed in the plant cells, causing them and their descendants to acquire resistance to concentrations of kanamycin which are normally toxic to plant cells.

Creation of NPT I Chimeric Gene

In an alternate preferred embodiment of this invention, a chimeric gene was created comprising (1) a NOS promoter region and 5' non-translated region, (2) a structural sequence which codes for NPT I, and (3) a NOS 3' non-translated region.

NPT I and NPT II are different and distinct enzymes with major differences in their amino acid sequences and substrate specificities. See, e.g., E. Beck et al, 1982. The relative stabilities and activities of these two enzymes in various types of plant cells are not yet fully understood, and NPT I may be preferable to NPT II for use in certain types of experiments and plant transformations.

A 1200 bp fragment containing an entire NPT I gene was obtained by digesting pACYC177 (Chang and Cohen, 1978) with the endonuclease, AvaII. The AvaII termini were converted to blunt ends with Klenow polymerase, and converted to BamHI termini using a synthetic linker. This fragment was inserted into a unique BamHI site in a pBR327-derived plasmid, as shown in FIG. **11**. The resulting plasmid was designated as pMON66.

Plasmid pMON57 (a deletion derivative of pBR327, as shown in FIG. **11**) was digested with AvaII. The 225 bp fragment of pMON57 was replaced by the analogous 225 bp AvaII fragment taken from plasmid pUC8 (Vieira and Messing, 1982), to obtain a derivative of pMON57 with no PstI cleavage sites. This plasmid was designated as pMON67.

Plasmid pMON58 (described previously and shown in FIG. 7) was digested with EcoRI and BamHI to obtain a 1300 bp fragment carrying the NOS promoter and the NPT II structural sequence. This fragment was inserted into pMON67 which had been digested with EcoRI and BamHI. The resulting plasmid was designated as pMON73, as shown in FIG. **12**.

pMON73 was digested with PstI and BamHI, and a 2.4 kb fragment was isolated containing a NOS promoter region and 5' non-translated region. Plasmid pMON66 (shown on FIG. **11**) was digested with XhoI and BamHI to yield a 950 bp fragment containing the structural sequence of NPT I. This fragment lacked about 30 nucleotides at the 5' end of the structural sequence. A synthetic linker containing the missing bases, having appropriate PstI and XhoI ends, was created. The pMON73 fragment, the pMON66 fragment, and the synthetic linker were ligated together to obtain plasmid pMON78, as shown in FIG. **13**. This plasmid contains the NOS promoter region and 5' non-translated region adjoined to the NPT I structural sequence. The ATG start codon was in the same position that the ATG start codon of the NOS structural sequence had occupied.

Plasmid pMON78 was digested with EcoRI and BamHI to yield a 1300 bp fragment carrying the chimeric NOS-NPT I regions. Double-stranded DNA from the M-2 clone (described previously and shown on FIG. **9**) was digested with EcoRI and BamHI, to yield a 280 bp fragment carrying a NOS 3' non-translated region with a poly-adenylation signal. The two fragments described above were ligated together to create the NOS-NPT I-NOS chimeric gene, which was inserted into plasmid pMON38 (described above) which had been digested with EcoRI. The two resulting plasmids, having chimeric gene inserts with opposite orientations, were designated as pMON106 and pMON107, as shown in FIG. **14**.

Either of plasmids pMON106 or pMON107 may be digested with EcoRI to yield a 1.6 kb fragment containing the chimeric NOS-NPT I-NOS gene. This fragment was inserted into plasmid pMON120 which had been digested with EcoRI and treated with alkaline phosphatase. The resulting plasmids, having inserts with opposite orientations, were designated as pMON130 and pMON131, as shown on FIG. **15**.

The NOS-NPT I-NOS chimeric gene was inserted into plant cells, which acquired resistance to kanamycin. This demonstrates expression of the chimeric gene in plant cells.

Creation of Chimeric Gene with Soybean Promoter

In an alternate preferred embodiment of this invention, a chimeric gene was created comprising (1) a promoter region and 5' non-translated region taken from a gene which naturally exists in soybean; this gene codes for the small subunit of ribulose-1,5-bis-phosphate carboxylase (sbss for soybean small subunit); (2) a structural sequence which codes for NPT II, and (3) a NOS 3' non-translated region.

The sbss gene codes for a protein in soybean leaves which is involved in photosynthetic carbon fixation. The sbss protein is the most abundant protein in soybean leaves (accounting for about 10% of the total leaf protein), so it is likely that the sbss promoter region causes prolific transcription.

There are believed to be approximately six genes encoding the ss RuBPCase protein in the soybean genome. One of the members of the ss RuBPCase gene family, SRS1, which is highly transcribed in soybean leaves, has been cloned and characterized. The promoter region, 5' nontranslated region, and a portion of the structural sequence are contained on a 2.1 kb EcoRI

fragment that was subcloned into the EcoRI site of plasmid pBR325 (Bolivar, 1978). The resultant plasmid, pSRS2.1, was a gift to Monsanto Company from Dr. R. B. Meagher, University of Georgia, Athens, Ga. The 2.1 kb EcoRI fragment from pSRS2.1 is shown on FIG. 16.

Plasmid pSRS2.1 was prepared from dam⁻ *E.coli* cells, and cleaved with MboI to obtain an 800 bp fragment. This fragment was inserted into plasmid pKC7 (Rao and Rogers, 1979) which had been cleaved with BglII. The resulting plasmid was designated as pMON121, as shown on FIG. 17.

Plasmid pMON121 was digested with EcoRI and BclI, and a 1200 bp fragment containing the sbss promoter region was isolated. Separately, plasmid pMON75 (described previously and shown on FIG. 9) was digested with EcoRI and BglII, and a 1250 bp fragment was isolated, containing a NPT II structural sequence and a NOS 3' non-translated region. The two fragments were ligated at the compatible BclI/BglII overhangs, to create a 2450 bp fragment containing sbss-NPT II-NOS chimeric gene. This fragment was inserted into pMON120 which had been cleaved with EcoRI, to create two plasmids having chimeric gene inserts with opposite orientations, as shown in FIG. 18. The plasmids were designated as pMON141 and pMON142.

The sbss-NPTII-NOS chimeric genes were inserted into several types of plant cells, causing the plant cells to acquire resistance to kanamycin.

This successful transformation proved that a promoter region from one type of plant can cause the expression of a gene within plant cells from an entirely different genus, family, and order of plants.

The chimeric sbss-NPT II-NOS gene also had another significant feature. Sequencing experiments indicated that the 800 bp MboI fragment contained the ATG start codon of the sbss structural sequence. Rather than remove this start codon, the Applicants decided to insert a stop codon behind it in the same reading frame. This created a dicistronic mRNA sequence, which coded for a truncated amino portion of the sbss polypeptide and a complete NPT II polypeptide. Expression of the NPT II polypeptide was the first proof that a dicistronic mRNA can be translated within plant cells.

The sbss promoter is contained in plasmid pMON154, described below. A culture of *E. coli* containing this plasmid has been deposited with the American Type Culture Center. This culture has been assigned accession number 39265.

Creation of BGH Chimeric Genes

In an alternate preferred embodiment of this invention, a chimeric gene was created comprising (1) a sbss promoter region and 5' non-translated region, (2) a structural sequence which codes for bovine growth hormone (BGH) and (3) a NOS 3' non-translated region. This chimeric gene was created as follows.

A structural sequence which codes for the polypeptide, bovine growth hormone, (see, e.g., Woychik et al, 1982) was inserted into a pBR322-derived plasmid. The resulting plasmid was designated as plasmid CP-1. This plasmid was digested with EcoRI and HindIII to yield a 570 bp fragment containing the structural sequence. Double stranded M-2 RF DNA (described previously and shown in FIG. 8) was cleaved with EcoRI and HindIII to yield a 290 bp fragment which contained the NOS 3' non-translated region with a poly-adenylation signal. The two fragments were ligated together and digested with EcoRI to create an 860 base pair fragment with EcoRI ends, which contained a BGH-coding structural sequence joined to the NOS 3' non-translated region. This fragment was introduced into plasmid pMON38, which had been digested with EcoRI and treated with alkaline phosphatase, to create a new plasmid, designated as pMON 108, as shown in FIG. 19.

A unique BglII restriction site was introduced at the 5' end of the BGH structural sequence by digesting pMON 108 with EcoRI to obtain the 860 bp fragment, and using Klenow polymerase to create blunt ends on the resulting EcoRI fragment. This fragment was ligated into plasmid N25 (a derivative of pBR327 containing a synthetic linker carrying BglII and XbaI cleavage sites inserted at the BamHI site), which had been cleaved with XbaI and treated with Klenow polymerase to obtain blunt ends (N25 contains a unique BglII site located 12 bases from the XbaI site). The resulting plasmid, which contained the 860 bp BGH-NOS fragment in the orientation shown in FIG. 20, was designated as plasmid N25-BGH. This plasmid contains a unique BglII cleavage site located about 25 bases from the 5' end of the BGH structural sequence.

Plasmid N25-BGH prepared from dam⁻ *E. coli* cells was digested with BglII and ClaI to yield an 860 bp fragment which contained the BGH structural sequence joined to the NOS 3' non-translated region. Separately, plasmid pMON121 (described previously and shown in FIG. 17) was prepared from dam⁻ *E. coli* cells and was digested with ClaI and BclI to create an 1100 bp fragment which contained the sbss promoter region. The fragments were ligated at their compatible BclI/BglII overhangs, and digested with ClaI to yield a ClaI fragment of about 2 kb containing the chimeric sbss-BGH-NOS gene. This fragment was inserted into pMON120 (described previously and shown in FIG. 10) which had been digested with ClaI. The resulting plasmids, containing the inserted chimeric gene in opposite orientations were designated pMON147 and pMON148, as shown in FIG. 21.

An alternate chimeric BGH gene was created which contained (1) a NOS promoter region and 5' non-translated region, (2) a structural sequence which codes for BGH, and (3) a NOS 3' non-translated region, by the following method, shown in FIG. 22.

Plasmid pMON76 (described above and shown in FIG. 9) was digested with EcoRI and BglII to obtain a 308 bp fragment containing a NOS promoter region and 5' non-translated region. Plasmid N25-BGH prepared from dam⁻ *E. coli* cells (described above and shown in FIG. 20) was digested with BglII and ClaI to obtain a 900 bp fragment containing a BGH structural sequence and a NOS 3' non-translated region. These two fragments were ligated together to obtain a chimeric NOS-BGH-NOS gene in a fragment with EcoRI and ClaI ends. This fragment was ligated with an 8 kb fragment obtained by digesting pMON120 with EcoRI and ClaI. The resulting plasmid, designated as pMON149, is shown in FIG. 22.

Creation of Chimeric NOS-EPSP-NOS Gene

In an alternate preferred embodiment, a chimeric gene was created comprising (1) a NOS promoter region and 5' non-translated region, (2) a structural sequence which codes for the *E. coli* enzyme, 5-enol pyru-

vyl shikimate-3-phosphoric acid synthase (EPSP synthase) and (3) a NOS 3' non-translated region.

EPSP synthase is believed to be the target enzyme for the herbicide, glyphosate, which is marketed by Monsanto Company under the registered trademark, "Roundup." Glyphosate is known to inhibit EPSP synthase activity (Amrhein et al, 1980), and amplification of the EPSP synthase gene in bacteria is known to increase their resistance to glyphosate. Therefore, increasing the level of EPSP synthase activity in plants may confer resistance to glyphosate in transformed plants. Since glyphosate is toxic to most plants, this provides for a useful method of weed control. Seeds of a desired crop plant which has been transformed to increase EPSP synthase activity may be planted in a field. Glyphosate may be applied to the field at concentrations which will kill all non-transformed plants, leaving the non-transformed plants unharmed.

An EPSP synthase gene may be isolated by a variety of means, including the following. A lambda phage library may be created which carries a variety of DNA fragments produced by HindIII cleavage of *E. coli* DNA. See, e.g., Maniatis et al, 1982.

The EPSP synthase gene is one of the genes which are involved in the production of aromatic amino acids. These genes are designated as the "aro" genes; EPSP synthase is designated as aroA. Cells which do not contain functional aro genes are designated as aro− cells. Aro− cells must normally be grown on media supplemented by aromatic amino acids. See Pittard and Wallis, 1966.

Different lambda phages which carry various HindIII fragments may be used to infect mutant *E. coli* cells which do not have EPSP synthase genes. The infected aro− cells may be cultured on media which does not contain the aromatic amino acids, and transformed aro+ clones which are capable of growing on such media may be selected. Such clones are likely to contain the EPSP synthase gene. Phage particles may be isolated from such clones, and DNA may be isolated from these phages. The phage DNA may be cleaved with one or more restriction endonucleases, and by a gradual process of analysis, a fragment which contains the EPSP synthase gene may be isolated.

Using a procedure similar to the method summarized above, the Applicants isolated an 11 kb HindIII fragment which contained the entire *E. coli* EPSP synthase gene. This fragment was digested with BglII to produce a 3.5 kb HindIII-BglII fragment which contained the entire EPSP synthase gene. This 3.5 kb fragment was inserted into plasmid pKC7 (Rao and Rogers, 1979) to produce plasmid pMON4, which is shown in FIG. **23**.

Plasmid pMON4 was digested with ClaI to yield a 2.5 kb fragment which contained the EPSP synthase structural sequence. This fragment was inserted into pBR327 that had been digested with ClaI, to create pMON8, as shown in FIG. **23**.

pMON8 was digested with BamHI and NdeI to obtain a 4.9 kb fragment. This fragment lacked about 200 nucleotides encoding the amino terminus of the EPSP synthase structural sequence.

The missing nucleotides were replaced by ligating a HinfI/NdeI fragment, obtained from pMON8 as shown in FIG. **24**, together with a synthetic oligonucleotide sequence containing (1) the EPSP synthase start codon and the first three nucleotides, (2) a unique BglII site, and (3) the appropriate BamHI and HinI ends. The resulting plasmid, pMON25, contains an intact EPSP synthase structural sequence with unique BamHI and BglII sites positioned near the start codon.

Double stranded M-2 DNA (described previously and shown in FIG. **8**) was digested with HindIII and EcoRI to yield a 290 bp fragment which contains the NOS 3' non-translated region and poly-adenylation signal. This fragment was introduced into a pMON25 plasmid that had been digested with EcoRI and HindIII to create a plasmid, designated as pMON146 (shown in FIG. **25**) which contains the EPSP structural sequence joined to the NOS 3' non-translated region.

pMON146 was cleaved with ClaI and BglII to yield a 2.3 kb fragment carrying the EPSP structural sequence joined to the NOS 3' non-translated region. pMON76 (described previously and shown in FIG. **9**) was digested with BglII and EcoRI to create a 310 bp fragment containing the NOS promoter region and 5' non-translated region. The above fragments were mixed with pMON120 (described previously and shown in FIG. **10**) that had been digested with ClaI and EcoRI, and the mixture was ligated. The resulting plasmid, designated pMON153, is shown in FIG. **26**. This plasmid contains the chimeric NOS-EPSP-NOS gene.

A plasmid containing a chimeric sbss-EPSP-NOS gene was prepared in the following manner, shown in FIG. **27**. Plasmid pMON146 (described previously and shown in FIG. **25**) was digested with ClaI and BglII, and a 2.3 kb fragment was purified. This fragment contained the EPSP synthase structural sequence coupled to a NOS 3' non-translated region with a poly-adenylation signal. Plasmid pMON121 (described above and shown in FIG. 17) was digested with ClaI and BclI, and a 1.1 kb fragment was purified. This fragment contains an sbss promoter region and 5' non-translated region. The two fragments were mixed and ligated with T4 DNA ligase and subsequently digested with ClaI. This created a chimeric sbss-EPSP-NOS gene, joined through compatible BglII and BclI termini. This chimeric gene with ClaI termini was inserted into plasmid pMON120 which had been digested with ClaI and treated with calf alkaline phosphatase (CAP). The mixture was ligated with T4 DNA ligase. The resulting mixture of fragments and plasmids was used to transform *E. coli* cells, which were selected for resistance to spectinomycin. A colony of resistant cells was isolated, and the plasmid in this colony was designated as pMON154, as shown in FIG. **27**.

A culture of *E. coli* containing pMON154 has been deposited with the American Type Culture Center. This culture has been assigned accession number 39265.

Means for Inserting Chimeric Genes Into Plant Cells

A variety of methods are known for inserting foreign DNA into plant cells. One such method, utilized by the Applicants, involved inserting a chimeric gene into Ti plasmids carried by *A. tumefaciens*, and co-cultivating the *A. tumefaciens* cells with plants. A segment of T-DNA carrying the chimeric gene was transferred into the plant genome, causing transformation. This method is described in detail in two separate, simultaneously-filed in two separate, simultaneously-filed applications entitled "Plasmids for Transforming Plant Cells," Ser. No. 458,411, and "Genetically Transformed Plants," Ser. No. 458,402. The contents of both of those applications are hereby incorporated by reference.

A variety of other methods are listed below. These methods are theoretically capable of inserting the chimeric genes of this invention into plant cells, although

the reported transformation efficiencies achieved to date by such methods have been low. The chimeric genes of this invention (especially those chimeric genes such as NPT I and NPT II, which may be utilized as selectable markers) are likely to facilitate research on methods of inserting DNA into plants or plant cells.

1. One alternate technique for inserting DNA into plant cells involves the use of lipid vesicles, also called liposomes. Liposomes may be utilized to encapsulate one or more DNA molecules. The liposomes and their DNA contents may be taken up by plant cells; see, e.g., Lurquin, 1981. If the inserted DNA can be incorporated into the plant genome, replicated, and inherited, the plant cells will be transformed.

To date, efforts to use liposomes to deliver DNA into plant cells have not met with great success (Fraley and Papahadjopoulos, 1981). Only relatively small DNA molecules have been transferred into plant cells by means of liposomes, and none have yet been expressed. However, liposome-delivery technology is still being actively developed, and it is likely that methods will be developed for transferring plasmids containing the chimeric genes of this invention into plant cells by means involving liposomes.

2. Other alternate techniques involve contacting plant cells with DNA which is complexed with either (a) polycationic substances, such as poly-L-ornithine (Davey et al, 1980), or (b) calcium phosphate (Krens et al, 1982). Although efficiencies of transformation achieved to date have been low, these methods are still being actively researched.

3. A method has been developed involving the fusion of bacteria, which contain desired plasmids, with plant cells. Such methods involve converting the bacteria into spheroplasts and converting the plant cells into protoplasts. Both of these methods remove the cell wall barrier from the bacterial and plant cells, using enzymic digestion. The two cell types can then be fused together by exposure to chemical agents, such as polyethylene glycol. See Hasezawa et al, 1981. Although the transformation efficiencies achieved to date by this method have been low, similar experiments using fusions of bacterial and animal cells have produced good results; see Rassoulzadegan et al, 1982.

4. Two other methods which have been used successfully to genetically transform animal cells involve (a) direct microinjection of DNA into animal cells, using very small glass needles (Capecchi, 1980), and (b) electric-current-induced uptake of DNA by animal cells (Wong and Neumann, 1982). Although neither of these techniques have been utilized to date to transform plant cells, they may be useful to insert chimeric genes of this invention into plant cells.

Use of Chimeric Genes to Identify Plant Regulators

The chimeric genes of this invention may be used to identify, isolate, and study DNA sequences to determine whether they are capable of promoting or otherwise regulating the expression of genes within plant cells.

For example, the DNA from any type of cell can be fragmented, using partial endonuclease digestion or other methods. The DNA fragments are mixed with multiple copies of a chimeric gene which has been cleaved at a unique cleavage site that is located in the 5' direction from the ATG start codon of the structural sequence. Preferably, the structural sequence, if properly transcribed, will be translated into a selectable marker, such as a polypeptide which confers resistance on the host to a selected antibiotic. The DNA mixture is ligated to form plasmids, and the plasmrids are used to transform plant cells which are sensitive to the selected antibiotic. The cells are cultured on media which contains an appropriate concentration of the selected antibiotic. Plant cells will survive and reproduce only if the structural sequence is transcribed and translated into the polypeptide which confers resistance to the antibiotic. This is presumed to occur only if the inserted DNA fragment performs the function of a gene promoter; the resistant colonies will be evaluated further to determine whether this is the case.

Using this technique, it is possible to evaluate the promoter regions of bacteria, yeast, fungus, algae, other microorganisms, and animal cells, to determine whether they also function as gene promoters in various types of plant cells. It is also possible to evaluate promoters from one type of plant in other types of plant cells. By using similar methods and varying the cleavage site in the chimeric gene, it is possible to evaluate the performance of any DNA sequence as a 5' non-translated region, a 3' non-translated region, a 3' non-translated region, or any type of other regulatory sequence.

If desired, a partial chimeric gene may be utilized in this method of evaluating the regulatory effects of various DNA sequences. For example, the NOS promoter region and/or the NOS 5' non-translated region may be deleted from the NOS-NPT II-NOS chimeric gene. This would create a chimeric gene having a unique cleavage site but no promoter region in front of an NPT II structural sequence.

In case the inserted DNA fragment contains a start codon which might (1) alter the reading frame of the structural sequence, or (2) alter the amino terminus of the polypeptide, it is possible to place an oligonucleotide between the cleavage site and the start codon of the structural sequence. The oligonucleotide would contain stop codons in all three reading frames. Therefore, if a start codon was included in the inserted DNA fragment, the gene would be a dicistronic gene. The first polypeptide would be terminated by whichever stop codon happened to be in the reading frame of the inserted start codon. The second start codon would begin the translation of a separate polypeptide, which would be the selectable marker enzyme.

Meaning of Various Phrases

A variety of phrases which are used in the claims must be defined and described to clarify the meaning and coverage of the claims.

The meaning of any particular term shall be interpreted with reference to the text and figures of this application. In particular, it is recognized that a variety of terms have developed which are used inconsistently in the literature. For example, a variety of meanings have evolved for the term "promoter," some of which include the 5' non-translated region and some of which do not. In an effort to avoid problems of interpretation, the Applicants have attempted to define various terms. However, such definitions are not presumed or intended to be comprehensive and they shall be interpreted in light of the relevant literature.

The term "chimeric gene" refers to a gene that contains at least two portions that were derived from different and distinct genes. As used herein, this term is limited to genes which have been assembled, synthesized, or otherwise produced as a result of man-made efforts,

and any genes which are replicated or otherwise derived therefrom. "Man-made efforts" include enzymatic, cellular, and other biological processes, if such processes occur under conditions which are caused, enhanced, or controlled by human effort or intervention; this excludes genes which are created solely by natural processes.

As used herein, a "gene" is limited to a segment of DNA which is normally regarded as a gene by those skilled in the art. For example, a plasmid might contain a plant-derived promoter region and a heterologous structural sequence, but unless those two segments are positioned with respect to each other in the plasmid such that the promoter region causes the transcription of the structural sequence, then those two segments would not be regarded as included in the same gene.

This invention relates to chimeric genes which have structural sequences that are "heterologous" with respect to their promoter regions. This includes at least two types of chimeric genes:

1. DNA of a gene which is foreign to a plant cell. For example, if a structural sequence which codes for mammalian protein or bacterial protein is coupled to a plant promoter region, such a gene would be regarded as heterologous.

2. A plant cell gene which is naturally promoted by a different plant promoter region. For example, if a structural sequence which codes for a plant protein is normally controlled by a low-quantity promoter, the structural sequence may be coupled with a prolific promoter. This might cause a higher quantity of transcription of the structural sequence, thereby leading to plants with higher protein content. Such a structural sequence would be regarded as heterologous with regard to the prolific promoter.

However, it is not essential for this invention that the entire structural sequence be heterologous with respect to the entire promoter region. For example, a chimeric gene of this invention may be created which would be translated into a "fusion protein", i.e., a protein comprising polypeptide portions derived from two separate structural sequences. This may be accomplished by inserting all or part of a heterologous structural sequence into the structural sequence of a plant gene, somewhere after the start codon of the plant structural sequence.

As used herein, the phrase, "a promoter region derived from a specified gene" shall include a promoter region if one or more parts of the promoter region were derived from the specified gene. For example, it might be discovered that one or more portions of a particular plant-derived promoter region (such as intervening region **8**, shown on FIG. **1**) might be replaced by one or more sequences derived from a different gene, such as the gene that contains the heterologous structural sequence, without reducing the expression of the resulting chimeric gene in a particular type of host cell. Such a chimeric gene would contain a plant-derived association region **2**, intervening region **4**, and transcription initiation sequence **6**, followed by heterologous intervening region **8**, 5′ non-translated region **10** and structural sequence **14**. Such a chimeric gene is within the scope of this invention.

As used herein, the phrase "derived from" shall be construed broadly. For example, a structural sequence may be "derived from" a particular gene by a variety of processes, including the following:

1. the gene may be reproduced by various means such as inserting it into a plasmid and replicating the plasmid by cell culturing, in vitro replication, or other methods, and the desired sequence may be obtained from the DNA copies by various means such as endonuclease digestion;
2. mRNA which was coded for by the gene may be obtained and processed in various ways, such as preparing complementary DNA from the mRNA and then digesting the cDNA with endonucleases;
3. the sequence of bases in the structural sequence may be determined by various methods, such as endonuclease mapping or the Maxam-Gilbert method. A strand of DNA which duplicates or approximates the desired sequence may be created by various methods, such as chemical synthesis or ligation of oligonucleotide fragments.
4. a structural sequence of bases may be deduced by applying the genetic code to the sequence of amino acid residues in a polypeptide. Usually, a variety of DNA structural sequences may be determined for any polypeptide, because of the redundancy of the genetic code. From this variety, a desired sequence of bases may be selected, and a strand of DNA having the selected sequence may be created.

If desired, any DNA sequence may be modified by substituting certain bases for the existing bases. Such modifications may be performed for a variety of reasons. For example, one or more bases in a sequence may be replaced by other bases in order to create or delete a cleavage site for a particular endonuclease. As another example, one or more bases in a sequence may be replaced in order to reduce the occurrence of "stem and loop" structures in messenger RNA. Such modified sequences are within the scope of this invention.

A structural sequence may contain introns and exons, such a structural sequence may be derived from DNA, or from an mRNA primary transcript. Alternately, a structural sequence may be derived from processed mRNA, from which one or more introns have been deleted.

The Applicants have deposited two cultures of *E. coli* cells containing plasmids pMON128 and pMON154 with the American Type Culture Collection (ATCC). These cells have been assigned ATCC accession numbers 39264 and 39265, respectively. The Applicants have claimed cultures of microorganisms having the "relevant characteristics" of either culture. As used herein, the "relevant characteristics" of a cell culture are limited to those characteristics which make the culture suitable for a use which is disclosed, suggested or made possible by the information contained herein. Numerous characteristics of the culture may be modified by techniques known to those skilled in the art; for example, the cells may be made resistant to a particular antibiotic by insertion of a particular plasmid or gene into the cells, or the pMON128 or pMON154 plasmids might be removed from the designated cells and inserted into a different strain of cells. Such variations are within the scope of this invention, even though they may amount to improvements, which undoubtedly will occur after more researchers gain access to these cell cultures.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific embodiments described herein. Such equivalents are within the scope of this invention.

EXAMPLES

Example 1 Creation of pMON1001

Fifty micrograms (ug) of lambda phage bbkan-1 DNA (Berg et al, 1975) were digested with 100 units of HindIII (all restriction endonucleases were obtained from New England Biolabs, Beverly, Mass., and were used with buffers according to the supplier's instructions, unless otherwise specified) for 2 hr at 37° C. After heat-inactivation (70° C., 10 min), the 3.3 kb Tn5 HindIII fragment was purified on a sucrose gradient. One ug of the purified HindIII fragment was digested with BamHI (2 units, 1 hr, 37° C.), to create a 1.8 kb fragment. The endonuclease was heat inactivated.

Plasmid pBR327 (Soberon et al, 1981), 1 ug, was digested with HindIII and BamHI (2 units each, 2 hr, 37° C.) Following digestion, the endonucleases were heat inactivated and the cleaved pBR327 DNA was added to the BamHI-HindIII Tn5 fragments. After addition of ATP to a concentration of 0.75 mM, 10 units of T4 DNA ligase (prepared by the method of Murray et al, 1979) was added, and the reaction was allowed to continue for 16 hours at 12°-14° C. One unit of T4 DNA ligase will give 90% circularization of one ug of HindIII-cleaved pBR327 plasmid in 5 minutes at 22° C.

The ligated DNA was used to transform $CaCl_2$-shocked *E. coli* C600 recA56 cells (Maniatis et al, 1982). After expression in Luria broth (LB) for 1 hour at 37° the cells were spread on solid LB media plates containing 200 ug/ml ampicillin and 40 ug/ml kanamycin. Following 16 hour incubation at 37° C., several hundred colonies appeared. Plasmid mini-prep DNA was prepared from six of these. (Ish-Horowicz and Burke, 1981). Endonuclease digestion showed that all six of the plasmids carried the 1.8 kb HindIII-BamHI fragment. One of those isolates was designated as pMON1001 as shown in FIG. **6**.

Example 2: Creation of pMON40

Five ug of plasmid pMON1001 (described in Example 1) was digested with SmaI. The reaction was terminated by phenol extraction, and the DNA was precipitated by ethanol. A BamHI linker CCGGATCCGG (0.1 ug), which had been phosphorylated with ATP and T4 polynucleotide kinase (Bethesda Research Laboratory, Rockville, Md.) was added to 1 ug of the pMON1001 fragment. The mixture was treated with T4 DNA ligase (100 units) for 18 hours at 14° C. After heating at 70° C. for 10 min to inactivate the DNA ligase, the DNA mixture was digested with BamHI endonuclease (20 units, 3 hours, 37° C.) and separated by electrophoresis on an 0.5% agarose gel. The band corresponding to the 4.2 kb SmaI-BamHI vector fragment was excised from the gel. The 4.2 kb fragment was purified by absorption on glass beads (Vogelstein and Gillespie, 1979), ethanol precipitated and resuspended in 20 ul of DNA ligase buffer with ATP. T4 DNA ligase (20 units) was added and the mixture was incubated for 1.5 hours at room temperature. The DNA was mixed with rubidium chloride-shocked in *E. coli* C600 cells for DNA transformation. (Maniatis et al, 1982). After expression for 1 hour at 37° C. in LB, the cells were spread on LB plates containing 200 ug/ml of ampicillin and 20 ug/ml kanamycin. The plates were incubated at 37° C. for 16 hours. Twelve ampicillin-resistant, kanamycin-resistant colonies were chosen, 2 ml cultures were grown, and mini-plasmid preparations were performed Endonuclease mapping of the plasmids revealed that ten of the twelve contained no SmaI site and a single BamHI site, and were of the appropriate size, 4.2 kb. The plasmid from one of the ten colonies was designated as pMON40, as shown in FIG. **6**.

Example 3: Creation of NOS Promoter Fragment

An oligonucleotide with the following sequence, 5'-TGCAGATTATTTGG-3', was synthesized (Beaucage and Carruthers, 1981, as modified by Adams et al, 1982). This oligonucleotide contained a ^{32}P radioactive label, which was added to the 5' thymidine residue by polynucleotide kinase.

An M113 mp7 derivative, designated as SlA, was given to Applicants by M. Bevan and M.-D. Chilton, Washington University, St. Louis, Mo. To the best of Applicants' knowledge and belief, the SlA DNA was obtained by the following method. A pTiT37 plasmid was digested with HindIII, and a 3.4 kb fragment was isolated and designated as the HindIII-23 fragment. This fragment was digested with Sau3a, to create a 344 bp fragment with Sau3a ends. This fragment was inserted into double-stranded, replicative form DNA from the M13 mp7 phage vector (Messing et al, 1981) which had been cut with BamHI. Two recombinant phages with 344 bp inserts resulted, one of which contained the anti-sense strand of the NOS promoter fragment. That recombinant phage was designated as SlA, and a clonal copy was given to the Applicants.

The Applicants prepared the single-stranded form of the SlA DNA (14.4 ug; 6 pmol), and annealed it (10 minutes at 70° C., then cooled to room temperature) with 20 pmol of the 14-mer oligonucleotide, mentioned above. The oligonucleotide annealed to the Sau3a insert at bases **286–300** as shown on FIGS. **4** and **5**.

200 ul of the SlA template and annealed oligonucleotide were mixed with the four dNTP's (present at a final concentration of 1 mM, 25 ul) and 50 ul of Klenow polymerase. The mixture incubated for 30 minutes at room temperature. During this period, the polymerase added dNTP's to the 3' end of the oligonucleogide. The polymerase was heat-inactivated (70° C., 3 min), and HaeIII (160 units) were added. The mixture was incubated (1 hour, 55° C.), the HaeIII was inactivated (70° C., 3 min), and the four dNTP's (1 mM, 12 ul) and T4 DNA polymerase (50 units) were added. The mixture was incubated (1 hour, 37° C.) and the polymerase was inactivated (70° C., 3 min). This yielded a fragment of about 570 bp. EcoRI (150 units) was added, the mixture was incubated (1 hour, 37° C.) and the EcoRI was inactivated (70° C., 3 min).

Aliquots of the mixture were separated on 6% acrylamide with 25% glycerol. Autoradiography revealed a radioactively labelled band about 310 bp in size. This band was excised. The foregoing procedure is indicated by FIG. **5**.

Example 4: Creation of pMON58

Five ug of plasmid pMON40 (described in Example 2) were digested with BglII (10 units, 1.5 hour, 37° C.), and the BglII was inactivated (70° C., 10 min). The four dNTP's (1mM, 5 ul) and Klenow polymerase (8 units) were added, the mixture was incubated (37° C., 40 min), and the polymerase was inactivated (70° C., 10 min). EcoRI (10 units) was added and incubated (1 hour, 37° C.), and calf alkaline phosphatase (CAP) was added and incubated (1 hour, 37° C.). A fragment of about 3.9 kb was purified on agarose gel using NA-45 membrane

(Scheicher and Scheull, Keene NH). The fragment (1.0 pM) was mixed with the NOS promoter fragment (0.1 pM), described in Example 3, and with T4 DNA ligase (100 units). The mixture was incubated (4° C., 16 hr). The resulting plasmids were inserted into *E. coli* cells, which were selected on media containing 200 ug/ml ampicillin. Thirty-six clonal Amp® colonies were selected, and mini-preps of plasmids were made from those colonies. The plasmid from one colony demonstrated a 308 bp EcoRI-BglII fragment, a new StuI cleavage site carried by the 308 bp NOS fragment, and a new PstI site . This plasmid was designated as pMON58, as shown in FIG. 7. pMON58 DNA was prepared as described above.

Example 5: Creation of pMON42

Plasmid pBR325-HindIII-23, a derivative of plasmid pBR325 (Bolivar, 1978) carrying the HindIII-23 fragment of pTiT37 (see FIG. 3) in the HindIII site, was given to Applicants by M. Bevan and M.-D. Chilton, Washington University, St. Louis, Mo. DNA of this plasmid was prepared and 30 ug were digested with HindIII (50 units) and BamHI (50 units). The 1.1 kb HindIII-BamHI fragment was purified by adsorption on glass beads (Vogelstein and Gillespie, 1979) after agarose gel electrophoresis. The purified fragment (0.5 ug) was added to 0.5 ug of the 2.9 kb HindIII-BamHI fragment of pBR327. After treatment with DNA ligase (20 units, 4 hours, 22° C.), the resulting plasmids were introduced to *E. coli* C600 cells. Clones resistant to ampicillin at 200 ug/ml were selected on solid media; 220 clones were obtained. Minipreps of plasmid DNA were made from six of these clones and tested with the presence of a 1.1 kb fragment after digestion with HindIII and BamHI. One plasmid which demonstrated the correct insert was designated pMON42. Plasmid pMON42 DNA was prepared as described in previous examples.

Example 6: Creation of M13 Clone M-2

Seventy-five ug of plasmid pMON42 (described in Example 5) prepared from dam⁻ *E. coli* cells were digested with RsaI and BamHI (50 units of each, 3 hours, 37° C.) and the 720 bp RsaI-BamHI fragment was purified using NA-45 membrane. Eight ug of the purified 720 bp BamHI-RsaI fragment were digested with MboI (10 min, 70° C.), the ends were made blunt by filling in with the large Klenow fragment of DNA polymerase I and the four dNTP's. Then 0.1 ug of the resulting DNA mixture was added to 0.05 ug of M13 mp8 previously digested with SmaI (1 unit, 1 hour 37° C.) and calf alkaline phosphatase (0.2 units). After ligation (10 units of T4 DNA ligase, 16 hours, 12° C.) and transfection of *E. coli* JM101 cells, several hundred recombinant phage were obtained. Duplex RF DNA was prepared from twelve recombinant, phage-carrying clones. The RF DNA (0.1 ug) was cleaved with EcoRI, (1 unit, 1 hour, 37° C.), end-labeled with ^{32}P-dATP and Klenow polymerase, and re-digested with BamHI (1 unit, 1 hour, 37° C.). The EcoRI and BamHI sites span the SmaI site. Therefore, clones containing the 260 bp MboI fragment could be identified as yielding a labelled 270 bp fragment after electrophoresis on 6% polyacrylamide gels and autoradiography. Four of the twelve clones carried this fragment. The orientation of the insert was determined by digestion of the EcoRI-cleaved, end-labeled RF DNA (0.1 ug) with HinfI (1 unit, 1 hour, 37° C.). HinfI cleaves the 260 bp MboI fragment once 99 bp from the 3' end of the fragment and again 42 bp from the end nearest the NOS coding region. Two clones of each orientation were obtained. One clone, digested as M-2 as shown in FIG. 8, contained the 260 bp fragment with the EcoRI site at the 3' end of the fragment. M-2 RF DNA was prepared using the procedures of Messing, et al 1981.

Example 7: Creation of pMON75 and pMON76

Fifty ug of M-2 RF DNA (described in Example 6) were digested with 50 units of EcoRI and 50 units of BamHI for 2 hours at 37°. The 270 bp fragment (1 ug) was purified using agarose gel and NA-45 membrane. Plasmid pMON58 (described in Example 4) was digested with EcoRI and BamHI (50 ug, 50 units each, 2 hours, 37° C.) and the 1300 bp fragment was purified using NA-45 membrane. The 270 bp EcoRI-BamHI (0.1 ug) and 1300 bp EcoRI-BamHI (0.5 ug) fragments were mixed, treated with T4 DNA ligase (2 units) for 12 hours at 14° C. After heating at 70° C. for 10 minutes to inactivate the ligase, the mixture was treated with EcoRI (10 units) for 1 hour at 37° C., then heated to 70° C. for 10 minutes to inactivate the EcoRI. This completed the assembly of a chimeric NOS-NPT II-NOS gene on a 1.6 kb fragment, as shown on FIG. 9.

Plasmid pMON38 is a clone of the pTiT37 HindIII-23 fragment inserted in the HindIII site of pBR327 (Soberon, et al 1980). pMON38 DNA (20 ug) was digested with EcoRI (20 units, 2 hours, 37° C.) and calf alkaline phosphatase (0.2 units, 1 hour, 37° C.) The pMON38 DNA reaction was extracted with phenol, precipitated with ethanol, dried and resuspended in 20 ul of 10 mM Tris-HCl, 1 mM EDTA, pH 8.

0.2 ug of the cleaved pMON38 DNA was added to the chimeric gene mixture described above. The mixture was treated with T4 DNA ligase (4 units, 1 hour, 22° C.) and mixed with Rb chloride-treated *E. coli* C600 recA56 cells to obtain transformation. After plating with selection for ampicillin-resistant (200 ug/ml) colonies, 63 potential candidates were obtained. Alkaline mini-preps of plasmid DNA were made from 12 of these and screened by restriction endonuclease digestion for the proper constructs. Plasmid DNA's that contained a 1.5 kb EcoRI fragment and a new BglII site were digested with BamHI to determine the orientation of the 1.5 kb EcoRI fragment. One of each insert orientation was picked. One plasmid was designated pMON75 and the other pMON76, as shown in FIG. 9. DNA from these plasmids were prepared as described in previous examples.

Example 8: Creation of plasmids pMON128 and pMON129

The 1.5 kb EcoRI fragment was excised by EcoRI digestion from either pMON75 or pMON76 and purified after agarose gel electrophoresis as described in previous examples. Five ug of DNA from plasmid pMON120 was digested with EcoRI and treated with calf alkaline phosphatase. After phenol deproteinization and ethanol precipitation, the EcoRI-cleaved pMON120 linear DNA was mixed with 0.5 ug of the 1.5 kb EcoRI chimeric gene fragment. The mixture was treated with 2 units of T4 DNA ligase for 1 hour at 22° C. After transformation of *E. coli* cells and (Maniatis, et al, 1982) selection of colonies resistant to spectinomycin (50 ug/ml), several thousand colonies appeared. Six of these were picked, grown, and plasmid mini-preps made. The plasmid DNA's were digested with EcoRI to check for the 1.5 kb chimeric gene insert and with

BamHI to determine the orientation of the insert. BamHI digestion showed that in pMON128 the chimeric gene was transcribed in the same direction as the intact nopaline synthase gene of pMON120. The orientation of the insert in pMON129 was opposite that in pMON128; the appearance of an additional 1.5 kb BamHI fragment in digests of pMON129 showed that plasmid pMON129 carried a tandem duplication of the chimeric NOS-NPT II-NOS gene, as shown in FIG. 10.

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We claim:

1. A chimeric gene capable of expressing a polypeptide in plant comprising in sequence:

a) a promoter region from a gene selected from the group consisting of an *Agrobacterium tumefaciens* opine synthase gene and a ribulose-1.5-bis-phosphate carboxylase small subunit gene;

b) a structural DNA sequence encoding a polypeptide that permits the selection of transformed plant cells containing said chimeric gene by rendering said plant cells resistant to an amount of an antibiotic that would be toxic to non-transformed plant cells, said structural DNA sequence being heterologous with respect to the promoter region; and

c) a 3' non-translated region of a gene naturally expressed in plants, said region encoding a signal sequence for polyadenylation of mRNA.

2. A gene of claim **1** in which the polypeptide renders transformed plant cells resistant to an amount of an aminoglycoside antibiotic that would be toxic to non-transformed plant cells.

3. A gene of claim **2** in which the polypeptide is a neomycin phosphotransferase.

4. A gene of claim **1** in which the 3' non-translated region is selected from a gene from the group consisting of the genes of the T-DNA region of *Agrobacterium tumefaciens.*

5. A gene of claim **1** in which the 3' non-translated region is from the nopaline synthase gene of *agrobacterium tumefaciens.*

6. A chimeric gene comprising in sequence:

(a) a promoter region from a gene selected from the group consisting of an *Agrobacterium tumefaciens* opine synthase gene and a ribulose-1.5-bis-phosphate carboxylase small subunit gene;

(b) a heterologous structural DNA sequence encoding a neomycin phosphotransferase; and

(c) a 3' non-translated region of a gene naturally expressed in plant cells, said region encoding a signal sequence for polyadenylation of mRNA.

7. A gene of claim **6** in which the 3' non-translated region is selected from a gene from the group consisting

of the genes of the T-DNA region of *Agrobacterium tumefaciens*.

8. A gene of claim **6** in which the 3' non-translated region is from the nopaline synthase gene of *Agrobacterium tumefaciens*.

9. A microorganism containing a chimeric gene of claim **1**.

10. A microorganism containing a chimeric gene of claim **2**.

11. A microorganism containing a chimeric gene of claim **6**.

12. A microorganism containing a chimeric gene of claim **3**.

13. A culture of microorganisms of claim **9**.

14. A culture of claim **13** in which the microorganism is *E. coli*.

15. A culture of claim **13** in which the microorganism is *Agrobacterium tumefaciens*.

16. A culture of claim **13** identified by ATCC Accession Number 39264.

17. A gene of claim **3** wherein said polypeptide is neomycin phosphotransferase I.

18. A gene of claim **3** wherein said polypeptide is neomycin phosphotransferase II.

19. A gene of claim **1** wherein said structural DNA sequence encodes for a neomycin phosphotransferase gene.

20. A gene of claim **19** wherein said structural DNA sequence encodes for a neomycin phosphotransferase I gene.

21. A gene of claim **19** wherein said structural DNA sequence encodes for a neomycin phosphotransferase II gene.

22. A gene of claim **6** wherein said heterologous structural DNA sequence is a neomycin phosphotransferase I gene.

23. A gene of claim **6** wherein said heterologous structural DNA sequence is a neomycin phosphotransferase II gene.

24. A microorganism containing a chimeric gene of claim **17**.

25. A microorganism containing a chimeric gene of claim **18**.

26. A microorganism containing a chimeric gene of claim **19**.

27. A microorganism containing a chimeric gene of claim **20**.

28. A microorganism containing a chimeric gene of claim **21**.

29. A microorganism containing a chimeric gene of claim **22**.

30. A microorganism containing a chimeric gene of claim **23**.

31. A microorganism identified by ATCC Accession Number 39264.

* * * * *

Exhibit 3 - U.S. Patent No. 5,352,605 to Fraley et al.

US005352605A

United States Patent [19]

Fraley et al.

[11] Patent Number: 5,352,605

[45] Date of Patent: Oct. 4, 1994

[54] **CHIMERIC GENES FOR TRANSFORMING PLANT CELLS USING VIRAL PROMOTERS**

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Related U.S. Application Data

[63] Continuation of Ser. No. 625,637, Dec. 7, 1990, abandoned, which is a continuation of Ser. No. 931,492, Nov. 17, 1986, abandoned, which is a continuation-in-part of Ser. No. 485,568, Apr. 15, 1983, abandoned, which is a continuation-in-part of Ser. No. 458,414, Jan. 17, 1983, abandoned.

[51] **Int. Cl.**[5] **C12N 5/00**; C12N 15/00; C07H 21/04

[52] **U.S. Cl.** **435/240.4**; 435/172.3; 435/320.1; 536/23.2; 536/24.1

[58] **Field of Search** 536/23.2, 24.1; 435/172.3, 240.4, 320.1; 800/205

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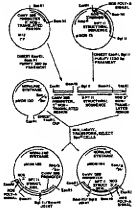
(List continued on next page.)

Primary Examiner—David T. Fox
Attorney, Agent, or Firm—Lawrence M. Lavin, Jr.; Dennis R. Hoerner, Jr.; Howard C. Stanley

[57] **ABSTRACT**

In one aspect the present invention relates to the use of viral promoters in the expression of chimeric genes in plant cells. In another aspect this invention relates to chimeric genes which are capable of being expressed in plant cells, which utilize promoter regions derived from viruses which are capable of infecting plant cells. One such virus comprises the cauliflower mosaic virus (CaMV). Two different promoter regions have been derived from the CaMV genome and ligated to heterologous coding sequences to form chimeric genes. These chimeric genes have been shown to be expressed in plant cells. This invention also relates to plant cells, plant tissue, and differentiated plants which contain and express the chimeric genes of this invention.

19 Claims, 10 Drawing Sheets



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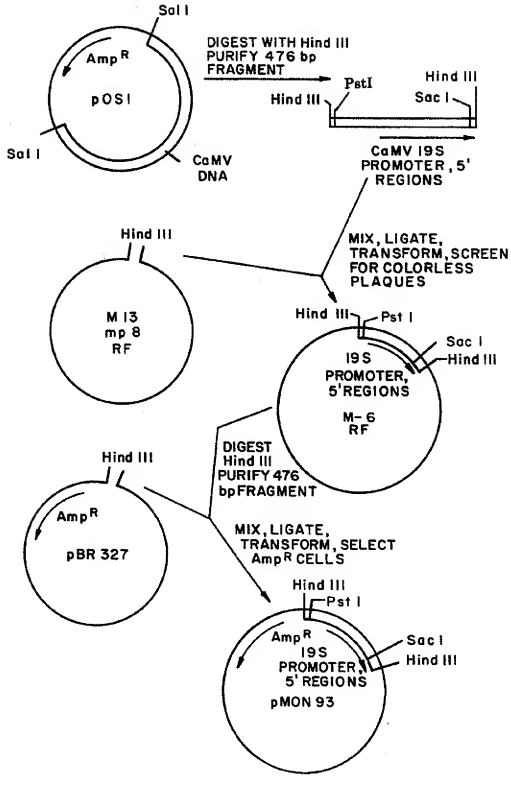

Sal I
Amp R
pOS1
Sal I
CaMV
DNA
DIGEST WITH Hind III
PURIFY 476 bp
FRAGMENT
PstI
Hind III
Hind III
Sac I
CaMV 19S
PROMOTER, 5'
REGIONS
MIX, LIGATE,
TRANSFORM, SCREEN
FOR COLORLESS
PLAQUES
Hind III
M 13
mp 8
RF
Hind III
Pst I
Sac I
Hind III
19S
PROMOTER,
5'REGIONS
M-6
RF
DIGEST
Hind III
PURIFY 476
bpFRAGMENT
Hind III
Amp R
pBR 327
MIX, LIGATE,
TRANSFORM, SELECT
Amp R CELLS
Hind III
Pst I
Amp R
19S
PROMOTER,
5' REGIONS
Sac I
Hind III
pMON 93

Figure 1

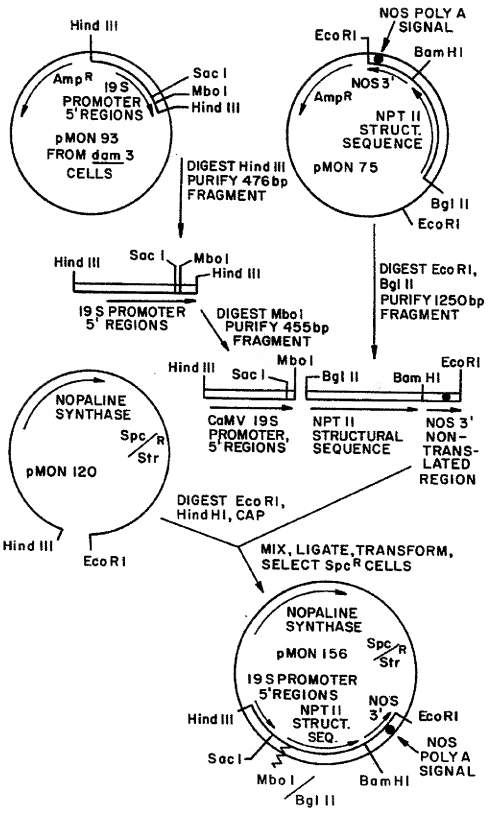

Hind III
Amp^R
19 S PROMOTER 5' REGIONS
Sac I
Mbo I
Hind III
pMON 93
FROM dam 3 CELLS
DIGEST Hind III
PURIFY 476bp FRAGMENT
NOS POLY A SIGNAL
EcoRI
BamHI
NOS 3'
AmpR
NPT II STRUCT. SEQUENCE
pMON 75
Bgl II
EcoRI
Hind III
Sac I
Mbo I
Hind III
19 S PROMOTER 5' REGIONS
DIGEST Mbo I
PURIFY 455bp FRAGMENT
DIGEST EcoRI, Bgl II
PURIFY 1250bp FRAGMENT
Hind III
Sac I
Mbo I
Bgl II
BamHI
EcoRI
CaMV 19S PROMOTER, 5' REGIONS
NPT II STRUCTURAL SEQUENCE
NOS 3' NON-TRANSLATED REGION
NOPALINE SYNTHASE
Spc/Str R
pMON 120
Hind III
EcoRI
DIGEST EcoRI, Hind III, CAP
MIX, LIGATE, TRANSFORM, SELECT Spc^R CELLS
NOPALINE SYNTHASE
pMON 156
Spc/Str R
19 S PROMOTER 5' REGIONS
NPT II STRUCT. SEQ.
NOS 3'
Hind III
EcoRI
Sac I
NOS POLY A SIGNAL
Mbo I
BamHI
Bgl II

Figure 2

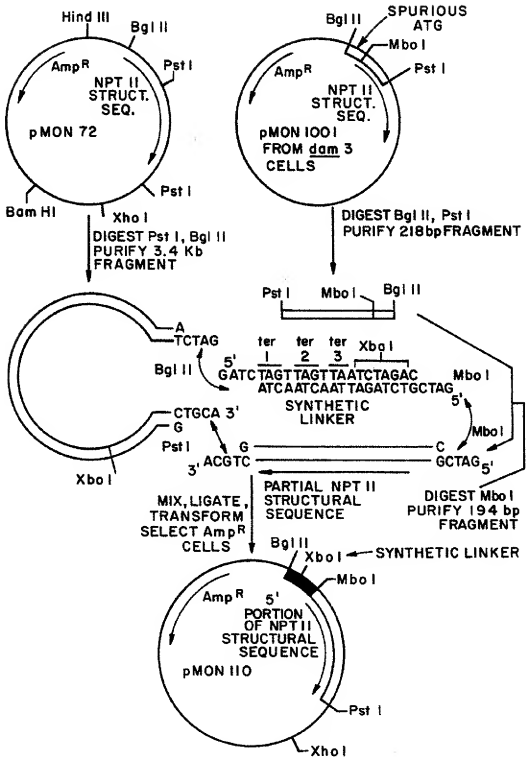

Hind III
Bgl II
Amp^R
NPT II STRUCT. SEQ.
Pst I
pMON 72
Pst I
Bam HI
Xho I
DIGEST Pst I, Bgl II PURIFY 3.4 Kb FRAGMENT
Bgl II
SPURIOUS ATG
Mbo I
Amp^R
Pst I
NPT II STRUCT. SEQ.
pMON 1001 FROM dam 3 CELLS
DIGEST Bgl II, Pst I PURIFY 218bp FRAGMENT
Pst I
Mbo I
Bgl II
A
TCTAG
ter 1
ter 2
ter 3
Xba I
Bgl II
5'
GATC TAGTTAGTTAATCTAGAC
ATCAATCAATTAGATCTGCTAG
Mbo I
5'
SYNTHETIC LINKER
CTGCA 3'
G
Pst I
G
C
3' ACGTC
GCTAG 5'
Mbo I
Xba I
PARTIAL NPT II STRUCTURAL SEQUENCE
DIGEST Mbo I PURIFY 194 bp FRAGMENT
MIX, LIGATE, TRANSFORM SELECT Amp^R CELLS
Bgl II
Xba I
SYNTHETIC LINKER
Mbo I
Amp^R
5'
PORTION OF NPT II STRUCTURAL SEQUENCE
pMON 110
Pst I
Xho I

Figure 3

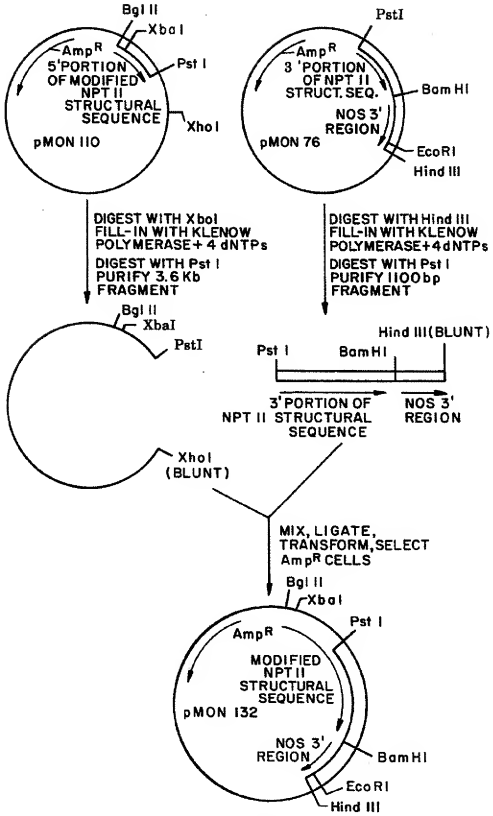

Bgl II
Xba I
AmpR
5'PORTION OF MODIFIED NPT II STRUCTURAL SEQUENCE
Pst I
Xho I
pMON 110
PstI
AmpR
3 'PORTION OF NPT II STRUCT. SEQ.
Bam HI
NOS 3' REGION
pMON 76
EcoRI
Hind III
DIGEST WITH Xbo I
FILL-IN WITH KLENOW POLYMERASE+ 4 dNTPs
DIGEST WITH Pst I
PURIFY 3.6 Kb FRAGMENT
DIGEST WITH Hind III
FILL-IN WITH KLENOW POLYMERASE+4 dNTPs
DIGEST WITH Pst I
PURIFY 1100 bp FRAGMENT
Bgl II
XbaI
PstI
Xho I
(BLUNT)
Hind III(BLUNT)
Pst I
BamHI
3'PORTION OF NPT II STRUCTURAL SEQUENCE
NOS 3' REGION
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
Bgl II
Xba I
Pst I
AmpR
MODIFIED NPT II STRUCTURAL SEQUENCE
pMON 132
NOS 3' REGION
Bam HI
EcoRI
Hind III

Figure 4

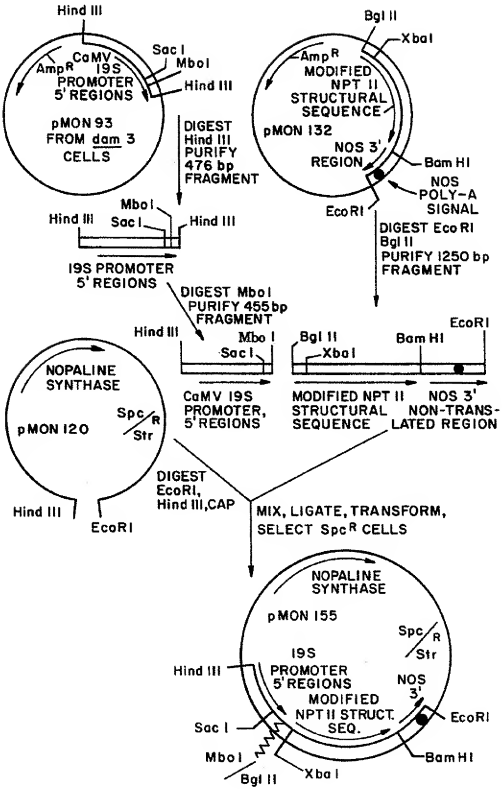

Hind III
CaMV
AmpR
19S
PROMOTER
5' REGIONS
Sac I
Mbo I
Hind III
pMON 93
FROM dam 3
CELLS
DIGEST
Hind III
PURIFY
476 bp
FRAGMENT
Bgl II
Xba I
AmpR
MODIFIED
NPT II
STRUCTURAL
SEQUENCE
pMON 132
NOS 3'
REGION
Bam HI
NOS
POLY-A
SIGNAL
EcoRI
DIGEST EcoRI
Bgl II
PURIFY 1250 bp
FRAGMENT
Mbo I
Hind III
Sac I
Hind III
19S PROMOTER
5' REGIONS
DIGEST Mbo I
PURIFY 455bp
FRAGMENT
EcoRI
Hind III
Mbo I
Sac I
Bgl II
Xba I
Bam HI
NOPALINE
SYNTHASE
pMON 120
Spc
Str
R
CaMV 19S
PROMOTER,
5' REGIONS
MODIFIED NPT II
STRUCTURAL
SEQUENCE
NOS 3'
NON-TRANS-
LATED REGION
DIGEST
EcoRI,
Hind III,CAP
Hind III
EcoRI
MIX, LIGATE, TRANSFORM,
SELECT Spc^R CELLS
NOPALINE
SYNTHASE
pMON 155
Spc
Str
R
19S
PROMOTER
5' REGIONS
Hind III
NOS
3'
MODIFIED
NPT II STRUCT.
SEQ.
EcoRI
Sac I
Mbo I
Bgl II
Xba I
Bam HI

Figure 5

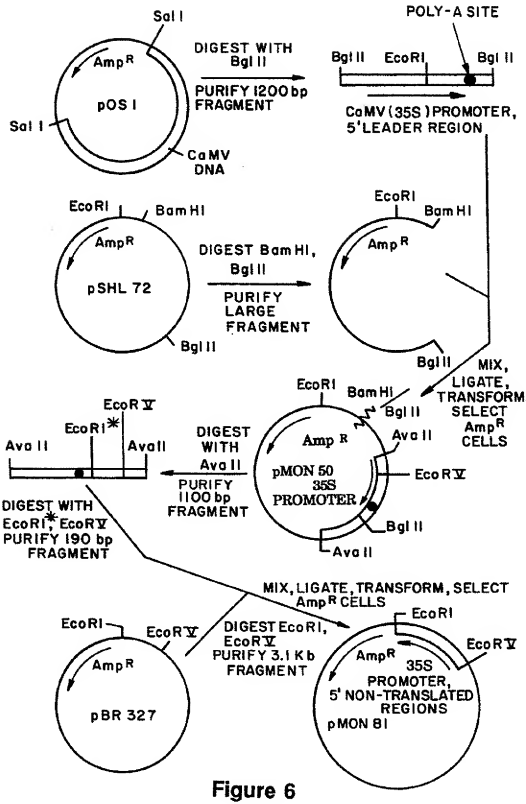

Sal I
Amp R
pOS I
Sal I
CaMV DNA
DIGEST WITH Bgl II
PURIFY 1200 bp FRAGMENT
POLY-A SITE
Bgl II EcoRI Bgl II
CaMV (35S) PROMOTER, 5'LEADER REGION
EcoRI BamHI
Amp R
pSHL 72
Bgl II
DIGEST BamHI, Bgl II
PURIFY LARGE FRAGMENT
EcoRI BamHI
Amp R
Bgl II
MIX, LIGATE, TRANSFORM, SELECT Amp R CELLS
EcoR V
EcoRI *
Ava II Ava II
DIGEST WITH Ava II
PURIFY 1100 bp FRAGMENT
EcoRI BamHI Bgl II
Amp R
Ava II
pMON 50 35S PROMOTER
EcoR V
Bgl II
Ava II
DIGEST WITH EcoRI * EcoR V
PURIFY 190 bp FRAGMENT
MIX, LIGATE, TRANSFORM, SELECT Amp R CELLS
EcoRI
EcoR V
Amp R
pBR 327
DIGEST EcoRI, EcoR V
PURIFY 3.1 Kb FRAGMENT
EcoRI
EcoR V
Amp R
35S PROMOTER, 5' NON-TRANSLATED REGIONS
pMON 81

Figure 6

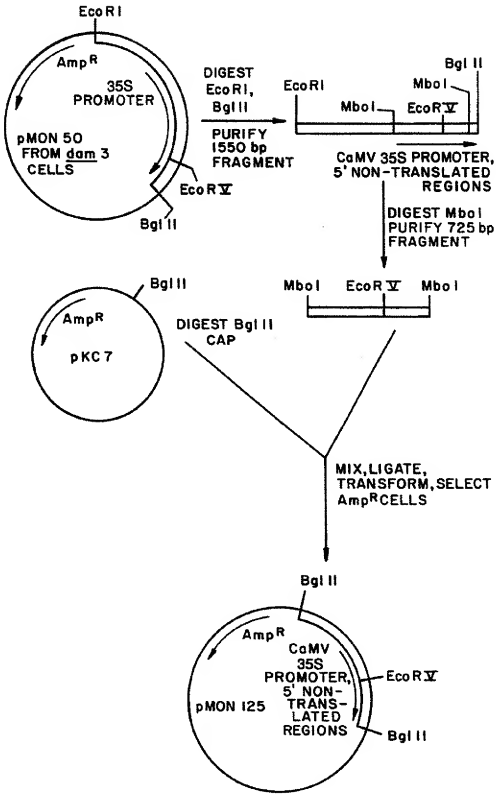

EcoRI
AmpR
35S PROMOTER
pMON 50 FROM dam 3 CELLS
EcoRV
BglII
DIGEST EcoRI, BglII
PURIFY 1550 bp FRAGMENT
EcoRI
MboI
BglII
MboI
EcoRV
CaMV 35S PROMOTER, 5' NON-TRANSLATED REGIONS
DIGEST MboI PURIFY 725 bp FRAGMENT
BglII
AmpR
pKC7
DIGEST BglII CAP
MboI
EcoRV
MboI
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
BglII
AmpR
CaMV 35S PROMOTER, 5' NON-TRANSLATED REGIONS
pMON 125
EcoRV
BglII

Figure 7

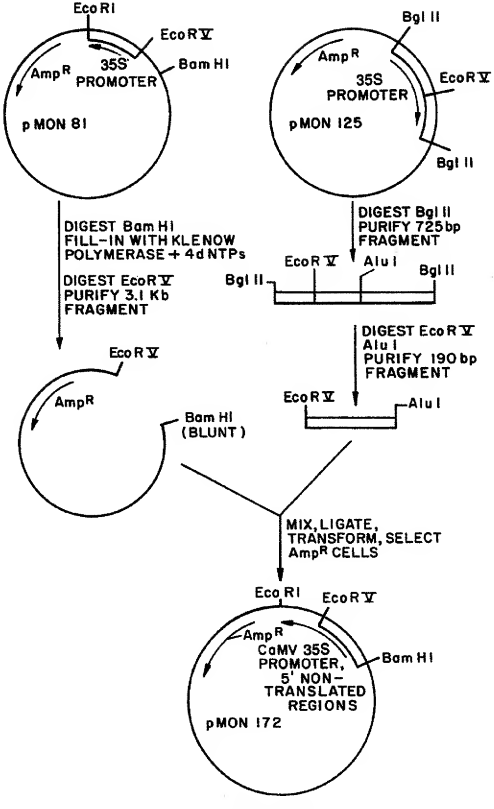

Eco RI
Eco R V
35S
PROMOTER
Amp R
Bam HI
p MON 81
Bgl II
Amp R
35S
PROMOTER
Eco R V
p MON 125
Bgl II
DIGEST Bam HI
FILL-IN WITH KLENOW
POLYMERASE + 4d NTPs
DIGEST Eco R V
PURIFY 3.1 Kb
FRAGMENT
DIGEST Bgl II
PURIFY 725 bp
FRAGMENT
Eco R V
Alu I
Bgl II
Bgl II
DIGEST Eco R V
Alu I
PURIFY 190 bp
FRAGMENT
Eco R V
Amp R
Bam HI
(BLUNT)
Eco R V
Alu I
MIX, LIGATE,
TRANSFORM, SELECT
Amp R CELLS
Eco RI
Eco R V
Amp R
CaMV 35S
PROMOTER,
5' NON-
TRANSLATED
REGIONS
Bam HI
pMON 172

Figure 8

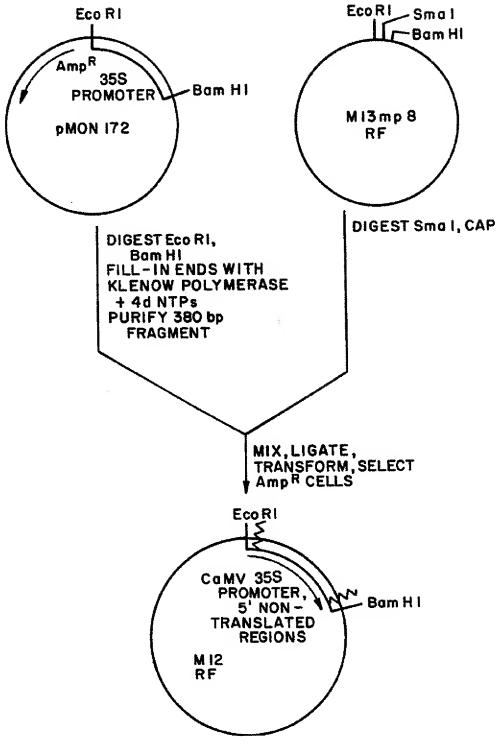

Eco RI
Amp^R
35S
PROMOTER
Bam HI
pMON 172
Eco RI
Sma I
Bam HI
M13mp 8
RF
DIGEST Eco RI,
Bam HI
FILL-IN ENDS WITH
KLENOW POLYMERASE
+ 4d NTPs
PURIFY 380 bp
FRAGMENT
DIGEST Sma I, CAP
MIX, LIGATE,
TRANSFORM, SELECT
Amp^R CELLS
Eco RI
CaMV 35S
PROMOTER,
5' NON-
TRANSLATED
REGIONS
Bam HI
M12
RF

Figure 9

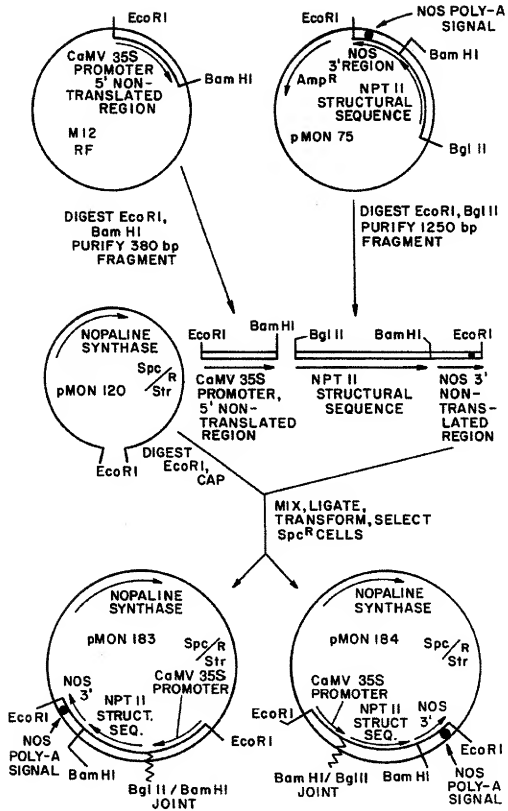

EcoRI
CaMV 35S PROMOTER 5' NON-TRANSLATED REGION
Bam HI
M12 RF
EcoRI
NOS POLY-A SIGNAL
NOS 3'REGION
Bam HI
AmpR
NPT II STRUCTURAL SEQUENCE
pMON 75
Bgl II
DIGEST EcoRI, Bam HI PURIFY 380 bp FRAGMENT
DIGEST EcoRI, Bgl II PURIFY 1250 bp FRAGMENT
NOPALINE SYNTHASE
Spc R
Str
pMON 120
EcoRI
Bam HI
Bgl II
Bam HI
EcoRI
CaMV 35S PROMOTER, 5' NON-TRANSLATED REGION
NPT II STRUCTURAL SEQUENCE
NOS 3' NON-TRANS-LATED REGION
DIGEST EcoRI, CAP
MIX, LIGATE, TRANSFORM, SELECT SpcR CELLS
NOPALINE SYNTHASE
pMON 183
Spc R
Str
NOS 3'
CaMV 35S PROMOTER
EcoRI
NPT II STRUCT. SEQ.
NOS POLY-A SIGNAL
Bam HI
EcoRI
Bgl II / Bam HI JOINT
NOPALINE SYNTHASE
pMON 184
Spc R
Str
CaMV 35S PROMOTER
EcoRI
NPT II STRUCT SEQ.
NOS 3'
EcoRI
Bam HI/ Bgl II JOINT
Bam HI
NOS POLY-A SIGNAL

Figure 10

CHIMERIC GENES FOR TRANSFORMING PLANT CELLS USING VIRAL PROMOTERS

RELATED APPLICATIONS

This is a File Wrapper continuation of application Ser. No. 07/625,637, filed Dec. 7, 1990, now abandoned, which is a continuation of U.S. Ser. No. 06/931,492, filed Nov. 17, 1986, now abandoned, which is a continuation-in-part of U.S. Ser. No. 06/485,568, filed Apr. 15, 1983, now abandoned, which is a continuation-in-part of U.S. Ser. No. 06/458,414, filed Jan. 17, 1983, now abandoned.

TECHNICAL FIELD

This invention is in the fields of genetic engineering and plant biology.

BACKGROUND ART

A virus is a microorganism comprising single or double stranded nucleic acid (DNA or RMA) contained within a protein (and possibly lipid) shell called a "capsid" or "coat". A virus is smaller than a cell, and it does not contain most of the components and substances necessary to conduct most biochemical processes. Instead, a virus infects a cell and uses the cellular processes to reproduce itself.

The following is a simplified description of how a DNA-containing virus infects a cell; RNA viruses will be disregarded in this introduction for the sake of clarity. First, a virus attaches to or enters a cell, normally called a "host" cell. The DNA from the virus (and possibly the entire viral particle) enters the host cell where it usually operates as a plasmid (a loop of extrachromosomal DNA). The viral DNA is transcribed into messenger RNA, which is translated into one or more polypeptides. Some of these polypeptides are assembled into new capsids, while others act as enzymes to catalyze various biochemical reactions. The viral DNA is also replicated and assembled with the capsid polypeptides to form new viral particles. These viral particles may be released gradually by the host cell, or they may cause the host cell to lyse and release them. The released viral particles subsequently infect new host cells. For more background information on viruses see, e.g., Stryer, 1981 and Matthews, 1970 (note: all references cited herein, other than patents, are listed with citations after the examples).

As used herein, the term "virus" includes phages and viroids, as well as replicative intermediates. As used herein, the phrases "viral nucleic acid" and DNA or RNA derived from a virus" are construed broadly to include any DNA or RNA that is obtained or derived from the nucleic acid of a virus. For example, a DNA strand created by using a viral RNA strand as a template, or by chemical synthesis to create a known sequence of bases determined by analyzing viral DNA, would be regarded as viral nucleic acid.

The host range of any virus (i.e., the variety of cells that a type of virus is capable of infecting) is limited. Some viruses are capable of efficient infection of only certain types of bacteria; other viruses can infect only plants, and may be limited to certain genera; some viruses can infect only mammalian cells. Viral infection of a cell requires more than mere entry of the viral DNA or RNA into the host cell; viral particles must be reproduced within the cell. Through various assays, those skilled in the art can readily determine whether any particular type of virus is capable of infecting any particular genus, species, or strain of cells. As used herein, the term "plant virus" is used to designate a virus which is capable of infecting one or more types of plant cells, regardless of whether it can infect other types of cells.

With the possible exception of viroids (which are poorly understood at present), every viral particle must contain at least one gene which can be "expressed" in infected host cells. The expression of a gene requires that a segment of DNA or RNA must be transcribed into or function as a strand of messenger RNA (mRNA), and the mRNA must be translated into a polypeptide. Most viruses have about 5 to 10 different genes, all of which are expressed in a suitable host cell.

In order to be expressed in a cell, a gene must have a promoter which is recognized by certain enzymes in the cell. Gene promoters are discussed in some detail in the parent application Ser. No. 458,414 cited above, the contents of which are incorporated herein by reference. Those skilled in the art recognize that the expression of a particular gene to yield a polypeptide is dependent upon two distinct cellular processes. A region of the 5' end of the gene called the promoter, initiates transcription of the gene to produce a mRNA transcript. The mRNA is then translated at the ribosomes of the cell to yield an encoded polypeptide. Therefore, it is evident that although the promoter may function properly, ultimate expression of the polypeptide depends at least in part on post-transcriptional processing of the mRNA transcript.

Promoters from viral genes have been utilized in a variety of genetic engineering applications. For example, chimeric genes have been constructed using various structural sequences (also called coding sequences) taken from bacterial genes, coupled to promoters taken from viruses which can infect mammalian cell(the most commonly used mammalian viruses are designated as Simian Virus 40 (SV40) and Herpes Simplex Virus (HSV)). These chimeric genes have been used to transform mammalian cells. See, e.g., Mulligan et al 1979; Southern and Berg 1982. In addition, chimeric genes using promoters taken from viruses which can infect bacterial cells have been used to transform bacterial cells; see, e.g., the phage lambda P_L promoter discussed in Maniatis et al, 1982.

Several researchers have theorized that it might be possible to utilize plant viruses as vectors for transforming plant cells. See, e.g., Hohn et al, 1982. In general, a "vector" is a DNA molecule useful for transferring one or more genes into a cell. Usually, a desired gene is inserted into a vector, and the vector is then used to infect the host cell.

Several researchers have theorized that it might be possible to create chimeric genes which are capable of being expressed in plant cells, by using promoters derived from plant virus genes. See, e.g., Hohn et al, 1982, at page 216.

However, despite the efforts of numerous research teams, prior to this invention no one had succeeded in (1) creating a chimeric gene comprising a plant virus promoter coupled to a heterologous structural sequence and (2) demonstrating the expression of such a gene in any type of plant cell.

CAULIFLOWER MOSAIC VIRUS (CaMV)

The entire DNA sequence of CaMV has been published. Gardner et al, 1981; Hohn et al, 1982. In its most

common form, the CaMV genome is about 8000 bp long. However, various naturally occurring infective mutants which have deleted about 500 bp have been discovered; see Howarth et al 1981. The entire CaMV genome is transcribed into a single mRNA, termed the "full-length transcript" having a sedimentation coefficient of about 35S. The promoter for the full-length mRNA (hereinafter referred to as "CaMV(35S)") is located in the large intergenic region about 1 kb counterclockwise from Gap 1 (see Guilley et al, 1982).

CaMV is believed to generate at least eight proteins; the corresponding genes are designated as Genes I through VIII. Gene VI is transcribed into mRNA with a sedimentation coefficient of 19S. The 19S mRNA is translated into a protein designated as P66, which is an inclusion body protein. The 19S mRNA is promoted by the 19S promoter, located about 2.5 kb counterclockwise from Gap 1.

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to the use of viral promoters in the expression of chimeric genes in plant cells. In another aspect this invention relates to chimeric genes which are capable of being expressed in plant cells, which utilize promoter regions derived from viruses which are capable of infecting plant cells. One such virus comprises the cauliflower mosaic virus (CaMV). Two different promoter regions have been derived from the CaMV genome and ligated to heterologous coding sequences to form chimeric genes. These chimeric genes have been proven to be expressed in plant cells. This invention also relates to plant cells, plant tissue (including seeds and propagules), and differentiated plants which have been transformed to contain viral promoters and express the chimeric genes of this invention, and to polypeptides that are generated in plant cells by the chimeric genes of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The figures herein are schematic representations; they have not been drawn to scale.

FIG. **1** represents the creation and structure of plasmid pMON93.

FIG. **2** represents the creation and structure of plasmid pMON156.

FIG. **3** represents the creation and structure of plasmid pMON110.

FIG. **4** represents the creation and structure of plasmid pMON132.

FIG. **5** represents the creation and structure of plasmid pMON155.

FIG. **6** represents the creation and structure of plasmid pMON81.

FIG. **7** represents the creation and structure of plasmid pMON125.

FIG. **8** represents the creation and structure of plasmid pMON172.

FIG. **9** represents the creation and structure of phage M12.

FIG. **10** represents the creation and structure of plasmids pMON183 and pMON184.

DETAILED DESCRIPTION OF THE INVENTION

In one preferred embodiment of this invention, a chimeric gene was created which contained the following elements:

1. a promoter region and a 5' non-translated region derived from the CaMV (19S) gene, which codes for the P66 protein;
2. a partial coding sequence from the CaMV (19S) gene, including an ATG start codon and several internal ATG sequences, all of which were in the same frame as a TGA termination sequence immediately inside the desired ATG start codon of the NPTII gene;
3. a structural sequence derived from a neomycin phosphotransferase II (NPTII) gene; this sequence was preceded by a spurious ATG sequence, which was in the same reading frame as a TGA sequence within the NPTII structural sequence; and,
4. a 3' non-translated region, including a poly-adenylation signal, derived from a nopaline synthase (NOS) gene.

This chimeric gene, referred to herein as the CaMV(19S)-NPTII-NOS gene, was inserted into plasmid pMON120 (described in the parent application, Ser. No. 458,414; ATCC accession number 39263) to create a plasmid designated as pMON156. Plasmid pMON156 was inserted into an *Agrobacterium tumefaciens* cell, where it formed a co-integrate Ti plasmid by means of a single crossover event with a Ti plasmid in the *A. tumefaciens* cell, using a method described in the parent application. The chimeric gene in the co-integrate plasmid was within a modified T-DNA region in the Ti plasmid, surrounded by left and right T-DNA borders. *A. tumefaciens* cells containing the co-integrate Ti plasmids with the CaMV(19S)-NPTII-NOS genes were used to infect plant cells, using a method described in the parent application. Some of the plant cells were genetically transformed, causing them to become resistant to an antibiotic (kanamycin) at concentrations which are toxic to untransformed plant cells.

A similar chimeric gene was created and assembled in a plasmid designated as pMON155. This chimeric gene resembled the gene in pMON156, with two exceptions:

1. an oligonucleotide linker having stop codons in all three reading frames was inserted between the CaMV(19S) partial structural sequence and the NPTII structural sequence; and,
2. the spurious ATG sequence on the 5' side of the NPTII structural sequence was deleted.

The construction of this chimeric gene is described in Example 2. This gene was inserted into *A. tumefaciens* cells and subsequently into plant cells. Its level of expression was apparently higher than the expression of the similar gene in pMON156, as assayed by growth on higher concentrations of kanamycin.

CREATION OF PLASMIDS pMON183 and 184; CaMV(35S)

In an alternate preferred embodiment of this invention, a chimeric gene was created comprising

(1) a promoter region which causes transcription of the 35S mRNA of cauliflower mosaic virus, CaMV(35S);

(2) a structural sequence which codes for NPTII; and

(3) a nopaline synthase (NOS) 3' non-translated region.

The assembly of this chimeric gene is described in Example 3. This gene was inserted into plant cells and it caused them to become resistant to kanamycin.

Petunia plants cannot normally be infected by CaMV. Those skilled in the art may determine through routine experimentation whether any particular plant

viral promoter (such as the CaMV promoter) will function at satisfactory levels in any particular type of plant cell, including plant cells that are outside of the normal host range of the virus from which the promoter was derived.

It is possible to regenerate genetically transformed plant cells into differentiated plants. One method for such regeneration was described in U.S. patent application entited "Genetically Transformed Plants", Ser. No. 458,402, now abandoned. That application was filed simultaneously with, and incorporated by reference into, the parent application of this invention. The methods of application Ser. No. 458,402, now abandoned, may be used to create differentiated plants (and their progeny) which contain and express chimeric genes having plant virus promoters.

It is possible to extract polypeptides generated in plant cells by chimeric genes of this invention from the plant cells, and to purify such extracted polypeptides to a useful degree of purity, using methods and substances known to those skilled in the art.

Those skilled in the art will recognize, or may ascertain using no more than routine experimentation, numerous equivalents to the specific embodiments described herein. Such equivalents are within the scope of this invention, and are covered by the claims below.

EXAMPLES

Example 1: Creation and Use of pMON156

Plasmids which contained CaMV DNA were a gift to Monsanto Company from Dr. R. J. Shepherd, University of California, Davis. To the best of Applicants' knowledge and belief, these plasmids (designated as pOS1) were obtained by inserting the entire genome of a CaMV strain designated as CM4-184 (Howarth et al, 1981) into the Sal I restriction site of a pBR322 plasmid (Bolivar et al, 1978). *E. coli* cells transformed with pOS1 were resistant to ampicillin (Amp^R) and sensitive to tetracycline (Tet^S).

Various strains of CaMV suitable for isolation of CaMV DNA which can be used in this invention are publicly available; see, e.g., ATCC Catalogue of Strains II, p. 387 (3rd edition, 1981).

pOS1 DNA was cleaved with HindIII. Three small fragments were purified after electrophoresis on an 0.8% agarose gel using NA-45 membrane (Schleicher and Schuell, Keene NH). The smallest fragment, about 500 bp in size, contains the 19S promoter. This fragment was further purified on a 6% acrylamide gel. After various manipulations which did not change the sequence of this fragment (shown in FIG. **1**), it was digested with MboI to created 455 bp HindIII-MboI fragment. This fragment was mixed with a 1250 bp fragment obtained by digesting pMON75 (described and shown in FIG. 9 of the parent application Ser. No. 458,414, now abandoned,) with BglII and EcoRI. This fragment contains the NPTII structural sequence and the NOS 3' non-translated region. The two fragments were ligated by their compatible MboI and BglII overhangs to create a fragment containing the CaMV(19S)-NPTII-NOS chimeric gene. This fragment was inserted into pMON120 (described and shown in FIG. **10** of the parent application, Ser. No. 458,414, now abandoned; ATCC accession number 39263) which had been cleaved with HindIII and EcoRI. The resulting plasmid was designated as pMON156, as shown in FIG. 2.

Plasmid pMON156 was inserted into *E. coli* cells and subsequently into *A. tumefaciens* cells where it formed a co-integrate Ti plasmid having the CaMV(19S)-NPTII-NOS chimeric gene surrounded by T-DNA borders. *A. tumefaciens* cells containing the co-integrate plasmids were co-cultivated with petunia cells. The foregoing methods are described in detail in a separate application, entitled "Plasmids for Transforming Plant Cells" Ser. No. 458,411, now abandoned, which was filed simultaneously with and incorporated by reference into parent application, Ser. No. 458,414, now abandoned.

The co-cultivated petunia cells were cultured on media containing kanamycin, an antibiotic which is toxic to petunia cells. Kanamycin is inactivated by the enzyme NPTII, which does not normally exist in plant cells. Some of the co-cultivated petunia cells survived and produced colonies on media containing up to 50 ug/ml kanamycin. This indicated that the CaMV(19S)-NPTII-NOS genes were expressed in petunia cells. These results were confirmed by Southern blot analysis of transformed plant cell DNA.

Example 2: Creation of pMON155

Plasmid pMON72 was obtained by inserting a 1.8 kb HindIII-BamHI fragment from bacterial transposon Tn5 (which contains an NPTII structural sequence) into a PstI- pBR327 plasmid digested with HindIII and BamHI. This plasmid was digested with BglII and PstI to remove the NPTII structural sequence.

Plasmid pMON1001 (described and shown in FIG. **6** of the parent application) from dam- cells was digested with BglII and PstI to obtain a 218 bp fragment with a partial NPTII structural sequence. This fragment was digested with MboI to obtain a 194 bp fragment.

A triple ligation was performed using (a) the large PstI-BglII fragment of pMON72; (b) PstI-MboI fragment from pMON1001; and (c) a synthetic linker with BglII and MboI ends having stop codons in all three reading frames. After transformation of *E. coli* cells and selection for ampicillin resistant colonies, plasmid DNA from Amp^R colonies was analyzed. A colony containing a plasmid with the desired structure was identified. This plasmid was designated pMON110, as shown on FIG. **3**.

In order to add the 3' end of the NPTII structural sequence to the 5' portion in pMON110, pMON110 was treated with XhoI. The resulting overhanging end was filled in to create a blunt end by treatment with Klenow polymerase and the four deoxy-nucleotide triphosphates (dNTP's), A, T, C, and G. The Klenow polymerase was inactivated by heat, the fragment was digested with PstI, and a 3.6 kb fragment was purified. Plasmid pMON76 (described and shown in FIG. **9** of the parent application) was digested with HindIII, filled in to create a blunt end with Klenow polymerase and the four dNTP's, and digested with PstI. An 1100 bp fragment was purified, which contained part of the NPTII structural sequence, and a nopaline synthase (NOS) 3' non-translated region. This fragment was ligated with the 3.6 kb fragment from pMON110. The mixture was used to transform *E. coli* cells; Amp R cells were selected, and a colony having a plasmid with the desired structure was identified. This plasmid was designated pMON132, as shown on FIG. **4**. Plasmid pMON93 (shown on FIG. **1**) was digested with HindIII, and a 476 bp fragment was isolated. This fragment was digested with MboI, and a 455 bp HindIII-MboI fragment was purified which contained the CaMV (19S) promoter region, and 5' non-translated region.

Plasmid pMON132 was digested with EcoRI and BglII to obtain a 1250 bp fragment with (1) the synthetic linker equipped with stop codons in all three reading frames; (2) the NPTII structural sequence; and (3) the NOS 3' non-translated region. These two fragments were joined together through the compatible MboI nd BglII ends to create a CaMV (19S)-NPTII-NOS chimeric gene.

This gene was inserted into pMON120, which was digested with HindIII and EcoRI, to create plasmid pMON155, as shown in FIG. 5.

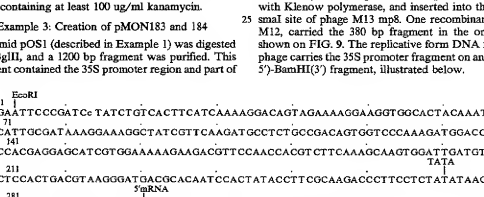
Plasmid pMON155 was inserted into *A. tumefaciens* GV3111 cells containing a Ti plasmid, pTiB6S3. The pMON155 plasmid formed a cointegrate plasmid with the Ti plasmid by means of a single crossover event. Cells which contain this co-integrate plasmid have been deposited with the American Type Culture Center, and have been assigned ATCC accession number 39336. A fragment which contains the chimeric gene of this invention can be obtained by digesting the co-integrate plasmid with HindIII and EcoRI, and purifying the 1.7 kb fragment. These cells have been used to transform petunia cells, allowing the petunia cells to grow on media containing at least 100 ug/ml kanamycin.

Example 3: Creation of pMON183 and 184

Plasmid pOS1 (described in Example 1) was digested with BglII, and a 1200 bp fragment was purified. This fragment contained the 35S promoter region and part of site of plasmid pKC7 (Rao and Rogers, 1979) to give plasmid pMON125, as shown in FIG. 7. The sequence of bases adjacent to the two MboI ends regenerates BglII sites and allows the 725 bp fragment to be excised with BglII.

To generate a fragment carrying the 35S promoter, the 725 bp BglII fragment was purified from pMON125 and was subsequently digested with EcoRV and AluI to yield a 190 bp fragment. Plasmid pMON81 was digested with BamHI, treated with Klenow polymerase and digested with EcoRV. The 3.1 kb EcoRV-BamHI(-blunt) fragment was purified, mixed with the 190 bp EcoRV-AluI fragment and treated with DNA ligase. Following transformation and selection of ampicillin-resistant cells, plasmid pMON172 was obtained which carries the CaMV(35S) promoter sequence on a 380 bp BamHI-EcoRI fragment, as shown on FIG. 8. This fragment does not carry the polyadenylation region for the 35S RNA. Ligation of the AluI end to the filled-in BamHI site regenerates the BamHI site.

To rearrange the restriction endonuclease sites adjacent to the CaMV(35S) promoter, the 380 bp BamHI-EcoRI fragment was purified from pMON172, treated with Klenow polymerase, and inserted into the unique smaI site of phage M13 mp8. One recombinant phage, M12, carried the 380 bp fragment in the orientation shown on FIG. 9. The replicative form DNA from this phage carries the 35S promoter fragment on an EcoRI(-5')-BamHI(3') fragment, illustrated below.



the 5' non-translated region. It was inserted into plasmid pSHL72 which had been digested with BamHI and BglII (pSHL72 is functionally equivalent to pAG060, described in Colbere-Garapin et al, 1981). The resulting plasmid was designated as pMON50, as shown on FIG. 6.

The cloned BglII fragment contains a region of DNA that acts as a polyadenylation site for the 35S RNA transcript. This polyadenylation region was removed as follows: pMON50 was digested with AvaII and an 1100 bp fragment was purified. This fragment was digested with EcoRI* and EcoRV. The resulting 190 bp EcoRV-EcoRI* fragment was purified and inserted into plasmid pBR327, which had been digested with EcoRI* and EcoRV. The resulting plasmid, pMON81, contains the CaMV 35S promoter on a 190 bp EcoRV-EcoRI* fragment, as shown in FIG. 6.

To make certain the entire promoter region of CaMV(35S) was present in pMON81, a region adjacent to the 5' (EcoRV) end of the fragment was inserted into pMON81 in the following way. Plasmid pMON50 prepared from dam- cells was digested with EcoRI and BglII and the resultant 1550 bp fragment was purified and digested with MboI. The resulting 725 bp MboI fragment was purified and inserted into the unique BglII

Plasmids carrying a chimeric gene CaMV(35S) promoter region-NPTII structural sequence-NOS 3' non-translated region) were assembled as follows. The 380 bp EcoRI-BamHI CaMV(35S) promoter fragment was purified from phage M12 RF DNA and mixed with the 1250 bp BglII-EcoRI NPTII-NOS fragment from pMON75. Joining of these two fragments through their compatible BamHI and BglII ends results in a 1.6 kb CaMV(35S)-NPTII-NOS chimeric genc. This gene was inserted into pMON120 at the EcoRI site in both orientations. The resultant plasmids, pMON183 and 184, appear in FIG. 10. These plasmids differ only in the direction of the chimeric gene orientation.

These plasmids were used to transform petunia cells, as described in Example 1. The transformed cells are capable of growth on media containing 100 ug/ml kanamycin.

COMPARISON OF CaMV(35S) AND NOS PROMOTERS

Chimeric genes carrying the nopaline synthase (NOS) promoter or the cauliflower mosaic virus full-length transcript promoter (CaMV(35S)) were con-

structed. In both cases, the promoters, which contain their respective 5' non-translated regions were joined to a NPTII coding sequence in which the bacterial 5' leader had been modified so that a spurious ATG translational initiation signal (Southern and Berg, 1982) has been removed.

Plasmid pMON200 is a derivative of previously described intermediate vector pMON120 (ATCC accession number 39263). pMON200 contains a modified chimeric nopaline synthase-neomycin phosphotransferasenopaline synthase gene (NOS/NPTII/NOS) which confers kanamycin (Km^R) resistance to the transformed plant. The modified chimeric Km^R gene lacks an upstream ATG codon present in the bacterial leader sequence and a synthetic multilinker with unique HindIII, XhoI, BglII, XbaI, ClaI and EcoRI restriction sites.

Plasmid pMON273 is a derivative of pMON200 in which the nopaline synthase promoter of the chimeric NOS-NPTII-NOS gene has been replaced with the CaMV(35S) promoter.

The CaMV(35S) promoter fragment was isolated from plasmid pOS-1, a derivative of pBR322 carrying the entire genome of CM4-184 as a SalI insert (Howarth et al., 1981). The CM4-184 strain is a naturally occurring deletion mutant of strain CM1841. The nucleotide sequence of the CM1841 (Gardner et al., 1981) and Cabb-S (Franck et al., 1980) strains of CaMV have been published as well as some partial sequence for a different CM4-184 clone (Dudley et al., 1982). The nucleotide sequences of the 35S promoter regions of these three isolates are essentially identical. In the following the nucleotide numbers reflects the sequence of Gardner et al. (1981). The 35S promoter was isolated as an AluI (n 7143)-EcoRI* (n 7517) fragment which was inserted first into pBR322 cleaved with BamHI, treated with the Klenow fragment of DNA polymerase I and then cleaved with EcoRI. The promoter fragment was then excised from pBR322 with BamHI and EcoRI, treated with Klenow polymerase and inserted into the SmaI site of M13 mp8 so that the EcoRI site of the mp8 multilinker was at the 5' end of the promoter fragment. Site directed mutagenesis (Zoller and Smith, 1982) was then used to introduce a G at nucleotide 7464 to create a BglII site. The 35S promoter fragment was then excised from the M13 as a 330 bp EcoRI-BglII site. The 35S promoter fragment was then excised from the M13 as a 330 bp EcoRI-BglII fragment which contains the 35S promoter, 30 nucleotides of the 5' non-translated leader but does not contain any of the CaMV translational initiators nor the 35S transcript polyadenylation signal that is located 180 nucleotides downstream from the start of transcription (Covey et al., 1981; Guilley et al., 1982). The CaMV(35S) promoter sequence described above is listed below.

pMON273 CaMV 35S Promoter and 5' Leader

```
   EcoRI
1  |                                                          70
GAATTCCCGATCs TATCTGTCACTTCATCAAAGGACAGTAGAAAAGGAAGGTGGCACTACAAATGCCAT
71                                                          140
CATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCAC
141                                                         210
CCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAT
                                                      TATA
211                                                         280
CTCCACTGACGTAAGGGATGACGCACAATCCACTATACCTTCGCAAGACCCTTCCTCTATATAAGGAAGT
                    5'mRNA                  BglII
281                                         |       334
TCATTTCATTTGGAGAGGACACGCTGAAATCACCAGTCTCTCTCTACAAGATCT
```

The 35S promoter fragment was joined to a 1.3 kb BglII-EcoRI fragment containing the Tn5 neomycin phosphotransferase II coding sequence modified so that the translational initiator signal in the bacterial leader sequence had been removed and the NOS 3' non-translated region and inserted into pMON120 to give pMON273.

These plasmids were transferred in *E. coli* strain JM101 and then mated into *Agrobacterium tumefaciens* strain GV3111 carrying the disarmed pTiB6S3-SE plasmid as described by Fraley et al. (1983).

Plant Transformation

Cocultivation of Petunia protoplasts with *A. tumefaciens*, selection of kanamycin resistant transformed callus and regeneration of transgenic plants was carried out as described in Fraley et al. (1984).

Preparation of DNAs

Plant DNA was extracted by grinding the frozen tissue in extraction buffer (50 mM TRIS-HCl pH 8.0, 50 mM EDTA, 50 mM NaCl, 400 ul/ml EtBr, 2% sarcosyl). Following low speed centrifugation, cesium chloride was added to the supernatant (0.85 gm/ml). The CsCl gradients were centrifuged at 150,000×g for 48 hours. The ethidium bromide was extracted with isopropanol, the DNA was dialyzed, and ethanol precipitated.

Southern Hybridization Analysis

10 ug of each plant DNA was digested, with BamHI for pMON200 plant DNAs and EcoRI for pMON273 plant DNAs. The fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to nitrocellulose (Southern, 1975). The blots were hybridized (50% formamide, 3xSSC, 5X denhardt's, 0.1% SDS and 20 ug/ml tRNA) with nick-translated pMON273 plasmid DNA for 48–60 hours at 42° C.

Preparation of RNA from Plant Tissue

Plant leaves were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The frozen tissue was added to a 1:1 mixture of grinding buffer and PCE (1% Tri-iso-propylnaphtaleasulfonic acid, 6% p-Aminosalicylic acid, 100 mM NaCl, 1% SDS and 50 mM 2-mercaptoethanol; PCI [phenol: chloroform: isoamyl alcohol (24:24:1)] and homogenized immediately with a polytron. The crude homogenate was mixed for 10 min and the phases separated by centrifugation. The aqueous phase then was re-extracted with an equal volume of PCI. The aqueous phase was ethanol precipitated with one tenth volume of 3M NaAcetate and 2.5 volumes of ethanol. The nucleic acid pellet was resuspended in water. An equal volume of 4M lithium chloride LiCl was added and the mix was placed on ice for 1 hour or overnight. Following cen-

trifugation, the pellet was resuspended in water the LiCl precipitation repeated 3 times. The final LiCl pellet was resuspended in water and ethanol precipitated.

Poly (A) containing RNA was isolated by passing total RNA over an Oligo d(T) cellulose Type III (Collaborative Research) column. Quantitation of the poly (A) containing RNA involved annealing an aliquot of the RNA to radio-labeled poly U [(uridylate 5,6-3H)-polyuridylic acid] (New England Nuclear), followed by RNase A treatment (10 ug per ml for 30 minutes at 37° C.). The reaction mix was spotted on DE-81 filter paper, washed 4× with 0.5M NaPhosphate (pH 7.5) and counted. Globin poly (A) containing RNA (BRL) was used as a standard.

Northern Hybridization Analysis

5 ug of poly (A) RNA from each plant source was treated with glyoxal and dimethysulfoxide (Maniatis, 1982). The RNAs were electrophoresed in 1.5% agarose gels (0.01M NaH_2HPO_4, pH 6.5) for 7 hours at 60 volts. The glyoxylated RNAs were electro-blotted (25 mM NaH_2PO_4/$NaHPO_4$, pH 6.5) for 16 hours at 125 amps from the gel to GeneScreen ® (New England Nuclear). The filters were hybridized as per manufacturer's instructions (50% formamide, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% ficoll, 5XSSC, 1.0% SDS, 100 u/ml tRNA and probe) for 48–60 hours at 42° C. with constant shaking. The nick-translated DNAs used as probes were the 1.3 kb BglII/EcoRI NPTII fragment purified from the pMON273 plasmid for detecting the NPTII transcript, and the petunia small subunit gene as an internal standard for comparing the amount of RNA per lane. The membranes were washed 2×100 ml of 2XSSC at room temperature for 5 minutes, 2×100 ml of 2XSSC/1.0% SDS at 65° C. for 30 minutes. The membranes were exposed to XAR-5 film with a DuPont intensifying screen at −80° C.

Neomycin Phosphotransferase Assay

The gel overlay assay was used to determine the steady state level of NPTII enzyme activity in each plant. Several parameters were investigated for optimizing the sensitivity of the assay in plant tissue. Early observations showed that the level of NPTII activity varied between leaves from different positions on the same plant. This variability was minimized when the plant extract was made from pooled tissue. A paper hole punch was used to collect 15 disks from both young and old leaves. Grinding the plant tissue in the presence of micro-beads (Ferro Corp) rather than glass beads increased the plant protein yield 4-fold.

To optimize detection of low levels of NPTII activity a saturation curve was prepared with 10–85 ug/lane of plant protein. For the pMON200 (NOS) plants, NPTII activity was not detectable at less than 50 ug/lane of total protein (2 hour exposure) while activity was detectable at 20 ug/lane for the pMON273 plants. There was a non-linear increase in NPTII activity for pMON200 NOS plants between 40 and 50 ug of protein per lane. This suggested that the total amount of protein may affect the stability of the NPTII enzyme. Supplementing plant cell extracts with 30–45 ug per lane of bovine serum albumin (BSA), resulted in a linear response; NPTII activity increased proportionately as plant protein levels increased. The addition of BSA appears to stabilize the enzyme, resulting in a 20-fold increase in the sensitivity of the assay. Experiments indicate that 25 ug/lane of pMON273 plant protein and 70 ug/lane of pMON200 plant protein was within the linear range of the assay in the presence of BSA. Elimination of SDS from the extraction buffer resulted in a 2-fold increase in assay sensitivity. Leaf disks were pooled from each plant for the assay. The tissue was homogenized with a glass rod in a microfuge tube with 150–200 ul of extraction buffer (20% glycerol, 10% β-mercaptoethanol, 125 mM Tris-HCl pH 6.8, 100 ug/ml bromophenol blue and 0.2% SDS). Following centrifugation in a microfuge for 20 minutes, total protein was determined using the Bradford assay. 25 ug of pMON273/3111SE plant protein or 70 ug of pMON200/3111SE plant protein, supplemented with BSA, was loaded on a native polyacrylamide gel as previously described. The polyacrylamide gel was equilibrated for 30 minutes in water and then 30 minutes in reaction buffer (67 mM TRIS-maleate pH 7.1, 43 mM $MgCl_2$, 400 mM NH_4Cl), transferred onto a glass plate, and overlaid with a 1.5% agarose gel. The overlay gel contained the neomycin phosphotransferase substrates: 450 uCi [γ-^{32}] ATP and 27 ug/ml neomycin sulfate (Sigma). After 1 hour at room temperature a sheet of Whatman P81 paper, two sheets of Whatman 3MM paper, a stack of paper towels and a weight were put on top of the agarose gel. The phosphorylated neomycin is positively charged and binds to the P81 phosphocellulose ion exchange paper. After blotting overnight, the P81 paper was washed 3× in 80° C. water, followed by 7 room temperature washes. The paper was air dried and exposed to XAR-5 film. Activity was quantitated by counting the ^{32}P-radioactivity in the NPTII spot. The NPTII transcript levels and enzyme activities in two sets of transgenic petunia plants were compared. In one set of plants (pMON273) the NPTII coding sequence is preceded by the CaMV(35S) promoter and leader sequences, in the other set of plants (PMON200) the NPTII coding region is preceded by the nopaline synthase promoter and leader sequences. The data indicates the pMON273 plants contain about a 30 fold greater level of NPTII transcript than the pMON200 plants, see Table I below.

TABLE I

QUANTITATION OF NPTII TRANSCRIPT LEVELS AND NPTII ACTIVITY IN pMON273 AND pMON200 PLANTS

Plant Number	Relative NPTII Transcript[a]	Relative NPTII Activity[b]
pMON 273		
3272	682	113
3271	519	1148
3349	547	447
3350	383	650
3343	627	1539
Average	551	779
pMON 200		
2782	0	0.22
2505	0	5.8
2822	0	0
2813	34	19
2818	0	1.0
3612	45	0.33
2823	97	23
Average	19	7
	~30-fold	~110-fold

TABLE I-continued

QUANTITATION OF NPTII TRANSCRIPT LEVELS AND NPTII ACTIVITY IN pMON273 AND pMON200 PLANTS

Plant Number	Relative NPTII Transcript[a]	Relative NPTII Activity[b]
	difference	difference

[a]Numbers derived from silver grain quantitation of autoradiogram. The RNA per lane was determined by filter hybridization to a petunia small subunit gene. The NPTII transcript values obtained with the NPTII probe were normalized for the amount of RNA in each lane.
[b]Numbers represent quantitation of NPT assay. Values were obtained by scintillation counting of 32-P-NPTII spots on the PE-81 paper used in the NPT assay as previously described. Values have been adjusted for the different amounts of protein loaded on the gels (25 ug) for pMON273 and 70 ug for pMON200 plants).

Consistent with this observation is the finding that the pMON273 leaf extracts have higher NPTII enzyme activity than the pMON200 leaf extracts. In several of the transgenic plants, there is a substantial variation in both RNA and enzyme levels which cannot be accounted for by the slight difference in gene copy number. Such "position effects" have been reported in transgenic mice and fruit flies and have not yet been adequately explained at the molecular level. Although, there is not a clear correlation between insert copy number and level of chimeric gene expression, the fact that 4 of the 7 pMON200 transgenic plants contain 2 copies of the NOS-NPTII-NOS gene would suggest that the differential expression of the CaMV(35S) promoter is actually slightly underestimated in these studies.

The constructs described in this comparative example have identical coding regions and 3' non-translated regions, indicating that the differences in the steady state transcript levels of these chimeric genes is a result of the 5' sequences.

COMPARISON OF CaMV19S AND CaMV(35S) PROMOTERS

Chimeric genes were prepared comprising either the CaMV19S or CaMV(35S) promoters. As in the above example, the promoters contained their respective 5' non-translated regions and were joined to a NPTII coding sequence in which the bacterial 5' leader had been modified to remove a spurious ATG translational initiation signal. The constructs tested were pMON203 and pMON204 containing the CaMV19S/NPTII/NOS gene and pMON273 containing the CaMV(35S)/NPTII/NOS gene.

Construction of pMON203

The CaMV 19S promoter fragment was isolated from plasmid pOS-1,a derivative of pBR322 carrying the entire genome of CM4-184 as a SalI insert (Howarth et al., 1981). The CM4-184 strain is a naturally occurring deletion mutant of strain CM1841. The references to nucleotide numbers in the following discussion are those for the sequence of CM1841 (Gardner et al., 1981). A 476 bp fragment extending from the HindIII site at bp 5372 to the HindIII site at bp 5848 was cloned into M13 mp8 for site directed mutagenesis (Zoller and Smith, 1982) to insert an XbaI (5'-TCTAGA) site immediately 5' of the first ATG translational initiation signal in the 19S transcript (Dudley et al., 1982). The resulting 400 bp HindIII-XbaI fragment was isolated and joined to the 1.3 kb XbaI-EcoRI fragment of pMON273 which carries the neomycin phosphotransferase II (NPTI') coding sequence modified so that the extra ATG translational initiation signal in the bacterial leader had been removed and the nopaline synthase 3' nontranslated region (NOS). The resulting 1.7 kb HindIII-EcoRI fragment was inserted into pMON120 between the EcoRI and HindIII sites to give pMON203. The complete sequence of the 19S promoter-NPTII leader is given below.

```
     HindIII
1|                                                              70
AAGCTTTAAAGCTGCAGAAAGGAATTACCACAGCAATGACAAAGAGACATTGGCGGTAATAAATACTATA
71                                                              140
AAGAAATTCAGTATTTATCTAACTCCTGTTCATTTTCTGATTAGGACAGATAATACTCATTTCAAGAGTT
141                                                             210
TTGTTAACCTTAATTACAAAGGAGATTCAAAACTTGGAAGAAACATCAGATGGCAAGCATGGCTTAGCCA
211                                                             280
CTATTCGTTTGATGTTGAACATATTAAAGGAACCGACAACCACTTTGCGGACTTCCTTTCAAGAGAATTC
281                                                             350
AATAAGGTTAATTCCTAATTGAAATCCGAAGATAAGATTCCCACACACTTGTGGCTGATATCAAAAAGGC
        TATA                    5'  mRNA
351                                                         402
TACTACCTATATAAACACATCTCTGGAGACTGAGAAAATCAGACCTCCAAGC
     XbaI                NPTII Initiator Signal
TCTAGACGATCGTTTCGC ATG
```

Construction of pMON204

The 400 bp HindIII-XbaI fragment containing the CaMV19S promoter was joined to a synthetic linker with the sequence:

```
          XbaI          BglII
5'-TCTAGACTCCTTACAACAGATCT
```

to add a BglII site to the 3' end of the promoter fragment. The HindIII-BglII fragment was joined to the 1.3 kb BglII-EcoRI fragment of pMON128 that contains the natural, unmodified NPTII coding sequence joined to the NOS 3' nontranslated signals and inserted into the EcoRI and HindIII sites of pMON120. The resulting plasmid is pMON204. The CaMV 19S promoter signals in this plasmid are identical to those in pMON203. The only difference is the sequence of the 5' nontranslated leader sequence which in pMON204 contains the extra ATG signal found in the bacterial leader of NPTII and contains extra bases from the synthetic linker and bacterial leader sequence.

Petunia leaf discs were transformed and plants regenerated as described above. The gel overlay assay was used to determine NPTII levels in transformants.

Quantitation was done by scintillation counting of ^{32}P-neomycin, the end product of neomycin phosphotransferase activity. The average NPTII enzyme level determined for CaMV(35S) (pMON273) plants was 3.6 times higher than that determined for CaMV(19S) (pMON203 & 204) plants.

QUANTITATION OF NPTII ACTIVITY LEVELS IN pMON203, pMON204, AND pMON273 PLANTS

Construct	Plant Number	Relative NPTII Activity[a]	Average
pMON203	4283	499,064	398,134
pMON203	4248	297,204	
			356,203
pMON204	4275	367,580	314,273
pMON204	4280	260,966	
pMON273	3350	1,000,674	1,302,731
pMON273	3271	1,604,788	

$$\frac{35s}{19s} \quad \frac{1,302,731}{356,203} = 3.6$$

[a]Numbers represent quantitation of NPT assay. Values were obtained by scintillation counting of ^{32}P-NPTII spots on the PE-81 paper used in the NPT assay as previously described.

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We claim:

1. A chimeric gene which is expressed in plant cells comprising a promoter from a cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV (35S) promoter isolated from CaMV protein-encoding DNA sequences and a CaMV (19S) promoter isolated from CaMV protein-encoding DNA sequences, and a structural sequence which is heterologous with respect to the promoter.

2. A chimeric gene of claim **1** in which the promoter is the CaMV(35S) promoter.

3. A chimeric gene of claim **1** in which the promoter is the CaMV(19S) promoter.

4. A plant cell which comprises a chimeric gene that contains a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV (35S) promoter and a CaMV (19S) promoter, wherein said promoter is isolated from CaMV protein-encoding DNA sequences, and a structural sequence which is heterologous with respect to the promoter.

5. A plant cell of claim **4** in which the promoter is the CaMV(35S) promoter.

6. A plant cell of claim **4** in which the promoter is the CaMV(19S) promoter.

7. An intermediate plant transformation plasmid which comprises a region of homology to an *Agrobacterium tumefaciens* vector, a T-DNA border region from *Agrobacterium tumefaciens* and a chimeric gene, wherein the chimeric gene is located between the T-DNA border and the region of homology, said chimeric gene comprising a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter and a CaMV(19S) promoter, and a structural sequence which is heterologous with respect to the promoter.

8. A plant transformation vector which comprises a disarmed plant tumor inducing plasmid of *Agrobacterium tumefaciens* and a chimeric gene, wherein the chimeric gene contains a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter and a CaMV(19S) promoter, and a structural sequence which is heterologous with respect to the promoter.

9. A plant transformation vector of claim **8** in which the promoter is the CaMV(35S) promoter.

10. A plant transformation vector of claim **8** in which the promoter is the CaMV(19S) promoter.

11. The chimeric gene of claim **1** comprising in the 5' to 3' direction:

(1) the CaMV(35S) promoter,
(2) a structural sequence encoding neomycin phosphotransferase II, and
(3) a 3' non-translated polyadenylation sequence of nopaline synthase.

12. The chimeric gene of claim **1** comprising in the 5' to 3' direction:

(1) the CaMV(19S) promoter,
(2) a structural sequence encoding neomycin phosphotransferase II, and
(3) a 3' non-translated polyadenylation sequence of nopaline synthase.

13. A DNA construct comprising:

(A) a CaMV promoter selected from the group consisting of (1) a CaMV 35S promoter isolated from CaMV protein-encoding DNA sequences and (2) a CaMV 19S promoter isolated from CaMV protein-encoding DNA sequences, and
(B) a DNA sequence of interest heterologous to (A), wherein (B) is under the regulatory control of (A) when said construct is transcribed in a plant cell.

14. A chimeric gene which is transcribed and translated in plant cells, said chimeric gene comprising a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of:

a) a CaMV 35S promoter region free of CaMV protein-encoding DNA sequences and
b) a CaMV 19S promoter region free of CaMV protein-encoding DNA sequences,

and a DNA sequence which is heterologous with respect to the promoter.

15. A chimeric gene which is expressed in plants cells comprising a promoter from a cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter region free of CaMV protein-encoding DNA sequences and a CaMV(19S) promoter

region free of CaMV protein-encoding DNA sequences, and a DNA sequence which is heterologous with respect to the promoter.

16. A chimeric gene which is transcribed in plants cells comprising a promoter from a cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter free of CaMV protein-encoding DNA sequences and a CaMV(19S) promoter free of CaMV protein-encoding DNA sequences, a DNA sequence which is heterologous with respect to the promoter and a 3' non-translated polyadenylation signal sequence.

17. A plant cell which comprises a chimeric gene where said chimeric gene comprises a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter and a CaMV(19S) promoter, wherein said promoter is free of CaMV protein-encoding DNA sequences, and a DNA sequence which is heterologous with respect to the promoter and a 3' non-translated polyadenylation signal sequence.

18. An intermediate plasmid of claim 7 in which the promoter is the CaMV(19S) promoter.

19. An intermediate plasmid of claim 7 in which the promoter is the CaMV(35S) promoter.

* * * * *

Exhibit 4 - U.S. Patent No. 5,850,015 to Bauer et al.

US005850015A

United States Patent [19]

Bauer et al.

[11] Patent Number: 5,850,015

[45] Date of Patent: Dec. 15, 1998

[54] **HYPERSENSITIVE RESPONSE ELICITOR FROM *ERWINIA CHRYSANTHEMI***

[75] Inventors: **David Bauer**; **Alan Collmer**, both of Ithaca, N.Y.

[73] Assignee: **Cornell Research Foundation, Inc.**, Ithaca, N.Y.

[21] Appl. No.: **484,358**

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[52] **U.S. Cl.** **800/205**; 435/69.1; 435/172.3; 435/320.1; 435/419; 536/23.7; 536/24.1

[58] **Field of Search** 800/205; 435/240.4, 435/69.1, 320.1, 172.3, 419; 536/23.7, 24.1

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[57] **ABSTRACT**

The present invention relates to an isolated protein or polypeptide corresponding to a protein or polypeptide in *Erwinia chrysanthemi* which elicits a hypersensitive response in plants. The encoding DNA molecule alone in isolated form or either in an expression system, a host cell, or a transgenic plant are also disclosed. Another aspect of the present invention relates to a method of imparting pathogen resistance to plants by transforming a plant with the DNA molecule of the present invention.

19 Claims, 6 Drawing Sheets

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1    AATGAGGAAACGAAATTATCCAAATTACGATCAAAGCCCACATCGGCCGTGATTTGGCCG
                M  Q  I  T  I  K  A  H  I  G  G  D  L  G

61   TCTCCGGTCTGGGGCTGGGTGCTCAGGGACTGAAAGGACTGAATTCCCCGCCTTCATCGC
     V  S  G  L  G  L  G  A  Q  G  L  K  G  L  N  S  A  A  S  S

121  TCGCTTCCAACCGTCGATAAACTGAGCAGCACCATCGATAAGTTGACCTCCCGCGTGAGTT
     L  G  S  S  V  D  K  L  S  S  T  I  D  K  L  T  S  A  L  T

181  CGATGATGTTTGGCGGCGCGCTGCGCAGGGGCTGGCGCCCAGCTCGAAGGGCCTGCGGA
     S  M  M  F  G  G  A  L  A  Q  G  L  G  A  S  S  K  G  L  G

241  TGAGCAATCAACTGGGCCAGTCTTTCGGCAATGGCGCGCAGGGTGCGAGCAACCTGCTAT
     M  S  N  Q  L  G  Q  S  F  G  N  G  A  Q  G  A  S  N  L  L

301  CCGTACCGAAATCGGGCGGCGATGCGTTGTCAAAAATGTTTGATAAAGCGCTGGACGATC
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     L  L  G  H  D  T  V  T  K  L  T  N  Q  S  N  Q  L  A  N  S
                                      4----> 439::Tn5-gusA1
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546::Tn5-gusA1 <------4
541  CTTCTCTGGGGGCAGGCGGCTTGCAGGGCCTGAGCGGCGCGGGTGCATTCAACCAGTTGG
     P  S  L  G  A  G  G  L  Q  G  L  S  G  A  G  A  F  N  Q  L

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     G  N  A  I  G  M  G  V  G  Q  N  A  A  L  S  A  L  S  N  V

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     S  T  H  V  D  G  N  N  R  H  F  V  D  K  E  D  R  G  M  A

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     K  E  I  G  Q  F  M  D  Q  Y  P  E  I  F  G  K  P  E  Y  Q

781  AAGATGGCTGGAGTTCGCCGAAGACGGACGACAAATCGTGGGCTAAAGCGCTGACTAAAC
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841  CGGATGATGACGGTATGACCGGCGCCAGCATGGACAAATTCGGTCAGGCGATGGGTATGA
     P  D  D  D  G  M  T  G  A  S  M  D  K  F  R  Q  A  M  G  M

901  TCAAAAGCGCGGTGGCGGGTGATACCGGCAATACCAACCTGAACCTGCGTGGCGCGGCG
     I  K  S  A  V  A  G  D  T  G  N  T  N  L  N  L  R  G  A  G

961  GTGCATCGCTGGGTATCGATGCGGCTGTCGTCGGCGATAAAATAGCCAACATGTCGCTGG
     G  A  S  L  G  I  D  A  A  V  V  G  D  K  I  A  N  M  S  L

1021 GTAAGCTGCCCAACGCCTGATAATCTGTGCTGGCCTGATAAAGCGGAAACGAAAAAAGAG
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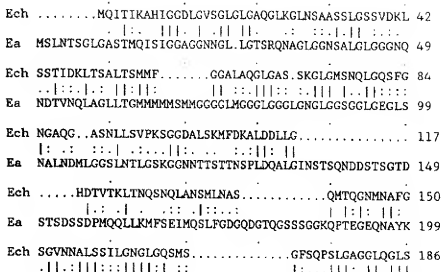
FIG. 1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Ech NGAQG..ASNLLSVPKSGGDALSKMFDKALDDLLG..... 117
Ea NALNDMLGGSLNTLGSKGGNNTTSTTNSPLDQALGINSTSQNDDSTSGTD 149
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Ea IGQFMDQYPEVFGKPQYQKGPGQEVKTDDKSWAKALSKPDDDGMTPASME 349
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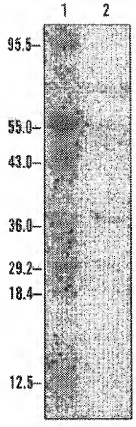
Ech LANA 340

FIG. 2



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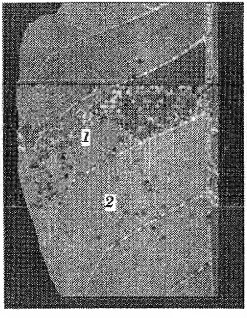

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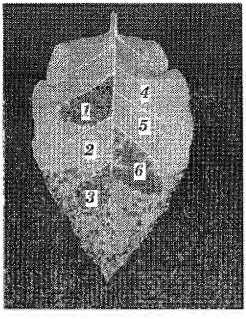

FIG. 5

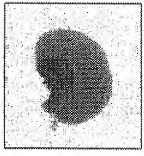

FIG. 6A

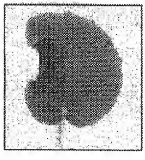

FIG. 6B

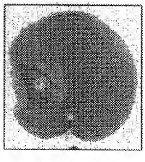

FIG. 6C

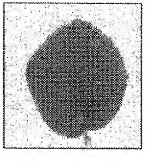

FIG. 6D

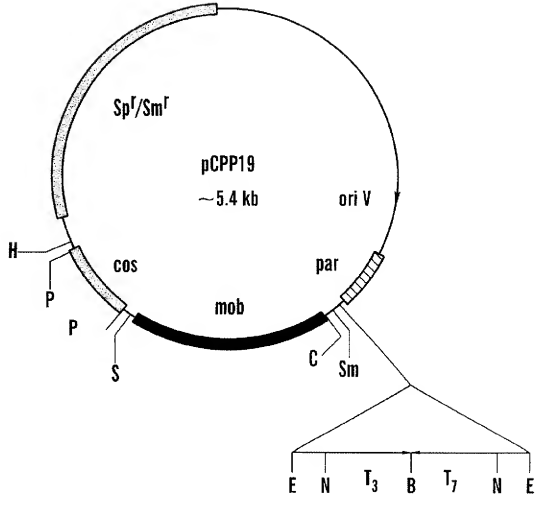

FIG. 7

HYPERSENSITIVE RESPONSE ELICITOR FROM *ERWINIA CHRYSANTHEMI*

This work was supported by NRI Competitive Grants Program/USDA grants 91-37303-6321 and 94-37303-0734.

FIELD OF THE INVENTION

The present invention relates to the Hypersensitive Response Elicitor from *Erwinia chrysanthemi* and its uses.

BACKGROUND OF THE INVENTION

Interactions between bacterial pathogens and their plant hosts generally fall into two categories: (1) compatible (pathogen-host), leading to intercellular bacterial growth, symptom development, and disease development in the host plant; and (2) incompatible (pathogen-nonhost), resulting in the hypersensitive response, a particular type of incompatible interaction occurring, without progressive disease symptoms. During compatible interactions on host plants, bacterial populations increase dramatically and progressive symptoms occur. During incompatible interactions, bacterial populations do not increase, and progressive symptoms do not occur.

The hypersensitive response is a rapid, localized necrosis that is associated with the active defense of plants against many pathogens (Kiraly, Z., "Defenses Triggered by the Invader: Hypersensitivity," pages 201–224 in: *Plant Disease: An Advanced Treatise*, Vol. 5, J. G. Horsfall and E. B. Cowling, ed. Academic Press New York (1980); Klement, Z., "Hypersensitivity," pages 149–177 in: *Phytopathogenic Prokaryotes*, Vol. 2, M. S. Mount and G. H. Lacy, ed. Academic Press, New York (1982)), which are hereby incorporated by reference). The hypersensitive response elicited by bacteria is readily observed as a tissue collapse if high concentrations (≧10^7 cells/ml) of a limited host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into the leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum) (Klement, Z., "Rapid Detection of Pathogenicity of Phytopathogenic Pseudomonads," *Nature* 199:299–300; Klement, et al., "Hypersensitive Reaction Induced by Phytopathogenic Bacteria in the Tobacco Leaf," *Phytopathology* 54:474–477 (1963); Turner, et al., "The Quantitative Relation Between Plant and Bacterial Cells Involved in the Hypersensitive Reaction," *Phytopathology* 64:885–890 (1974); Klement, Z., "Hypersensitivity," pages 149–177 in *PhytoDathogenic Prokaryotes*, Vol. 2., M. S. Mount and G. H. Lacy, ed. Academic Press, New York (1982), which are hereby incorporated by reference). The capacities to elicit the hypersensitive response in a nonhost and be pathogenic in a host appear linked. As noted by Klement, Z., "Hypersensitivity," pages 149–177 in *Phytopathogenic Prokaryotes*, Vol. 2., M. S. Mount and G. H. Lacy, ed. Academic Press, New York which is incorporated by reference, these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the hypersensitive response or pathogenesis is dependent on a common set of genes, denoted hrp (Lindgren, P. B., et al., "Gene Cluster of *Pseudomonas syringae* pv. 'phaseolicola' Controls Pathogenicity of Bean Plants and Hypersensitivity on Nonhost Plants," *J. Bacteriol.* 168:512–22 (1986); Willis, D. K., et al., "hrp Genes of Phytopathogenic Bacteria," *Mol. Plant-Microbe Interact.* 4:132–138 (1991), which are hereby incorporated by reference). Consequently, the hypersensitive response may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The hrp genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis, D. K., et al., "hrp Genes of Phytopathogenic Bateria," *Mol. Plant-Microbe Interact.* 4:132–138 (1991); Bonas, U., "hrp Genes of Phytopathogenic Bacteria," pages 79–98 in: *Current Topics in Microbiology and Immunology: Bacterial Pathogenesis of Plants and Animals—Molecular and Cellular Mechanisms*, J. L. Dangl, ed. Springer-Verlag, Berlin (1994), which are hereby incorporated by reference). Several hrp genes encode components of a protein secretion pathway similar to one used by Yersinia, Shigella, and Salmonella spp. to secrete proteins essential in animal diseases (Van Gijsegem, et al., "Evolutionary Conservation of Pathogenicity Determinants Among Plant and Animal Pathogenic Bacteria," *Trends Microbiol.* 1:175–180 (1993), which is incorporated by reference). In *E. amylovora, P. syringae,* and *P. solanacearum*, hrp genes have been shown to control the production and secretion of glycine-rich, protein elicitors of the hypersensitive response (He, S.Y., et al. "Pseudomonas Syringae pv. Syringae HarpinPss: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," *Cell* 73:1255–1266 (1993), Wei, Z.-H., et al., "Hrpl of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family," *J. Bacteriol.* 175:7958–7967 (1993); Arlat, M. et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," *EMBO J.* 13:543–553 (1994), which are hereby incorporated by reference).

The first of these proteins was discovered in E. amylovora Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei, Z.- M., et al, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," *Science* 257:85–88 (1992), which is incorporated by reference). Mutations in the encoding hrpN gene revealed that harpin is required for *E. amylovora* to elicit a hypersensitive response in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the hypersensitive response in leaves of tobacco, which is not a host of that strain (Arlat, et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of Pseudomonas solanacearum," *EMBO J.* 13:543–553 (1994), which is incorporated by reference). However, *P. solanacearum* popA mutants still elicit the hypersensitive response in tobacco and incite disease in tomato. Thus, the role of these glycine-rich hypersensitive response elicitors can vary widely among gram-negative plant pathogens.

E. chrysanthemi is unlike the bacterial pathogens that typically elicit the hypersensitive response, because it has a wide host range, rapidly kills and macerates host tissues, and secretes several isozymes of the macerating enzyme pectate lyase (Barras, F., et al., "Extracellular Enzymes and Pathogenesis of Soft-rot Erwinia," *Annu. Rev. Phytopathol.* 32:201–234 (1994), which is incorporated by reference). Nevertheless, pectic enzyme secretion pathway mutants of *E. chrysanthemi* EC16 cause a typical hypersensitive response (Bauer, D. W., et al., "*Erwinia chrysanthemi* hrp Genes and Their Involvement in Soft Rot Pathogenesis and Elicitation of the Hypersensitive Response," *Mol. Plant-Microbe Interact.* 7:573–581 (1994), which is incorporated by reference). Furthermore, elicitation of the hypersensitive response by *E. chrysanthemi* is dependent on a hrp gene that is conserved in *E. amylovora* and *P. syringae* and functions

in the secretion of the *E. amylovora* harpin (Wei, Z.-H., et al., "Hrpl of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family," *J. Bacteriol.* 175:7958–7967 (1993); Bauer, D. W., et al., "*Erwinia chrysanthemi* hrp Genes and Their Involvement in Soft Rot Pathogenesis and Elicitation of the Hypersensitive Response," *Mol. Plant-Microbe Interact.* 7:573–581 (1993), which are hereby incorporated by reference).

SUMMARY OF THE INVENTION

The present invention relates to an isolated protein or polypeptide corresponding to a protein or polypeptide in *Erwinia chrysanthemi* which elicits a hypersensitive response in plants. The encoding DNA molecule in isolated form or in either an expression system, a host cell, or a transgenic plant is also disclosed.

Another aspect of the present invention relates to a method of imparting pathogen resistance to plants by transforming them with a DNA molecule encoding the protein or polypeptide capable of eliciting a hypersensitive response in plants and corresponding to a protein or polypeptide in *Erwinia chrysanthemi* which elicits hypersensitive response in plants.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the DNA sequence of $hrpN_{Ech}$ and predicted amino acid sequence of its product. Underlined are the putative ribosome binding site, the N-terminal amino acids confirmed by sequencing the product of pCPP2172, and a potential rho-independent transcription terminator. The location and orientation of two Tn5-gusA1 insertions are also indicated and are numbered according to their location in the $hrpN_{Ech}$ open reading frame. The accession number for hrpN is L39897.

FIG. **2** compares the predicted amino acid sequences of $HrpN_{Ech}$ and $HrpN_{Ea}$. The predicted amino acid sequences of the *Erwinia chrysanthemi* ("*Ech*") and *E. amylovora* ("*Ea*") hrpN products were aligned by the Gap Program of the Genetics Computer Group Sequence Analysis Software Package (Devereaux, J., et al., "A Comprehensive Set of Sequence Analysis Programs for the VAX," *Gene* 12.387–395 (1984)). Two dots denote higher similarity than one dot.

FIG. **3** is an SDS polyacrylamide gel of purified $HrpN_{Ech}$. Purified $HrpN_{Ech}$ was solubilized in SDS loading buffer, electrophoresed through a 12% polyacrylamide gel, and stained with Coomassie Brilliant Blue. Lane 1, molecular weight markers (mid range markers from Diversified Biotech, Boston, Mass.) with size in kD shown to the left; lane 2, $HrpN_{Ech}$.

FIG. **4** shows the response of tobacco leaf tissue to purified $HrpN_{Ech}$. Leaf panel 1 was infiltrated with a suspension of purified $HrpN_{Ech}$ at a concentration of 336 μg/ml of 5 mM MES (morpholinoethanesulforic acid), pH 6.5. Panel 2 was infiltrated with buffer alone. The tissue in panel 1 collapsed 18 hrs. later. The leaf was photographed 24 hrs. after infiltration using a cross-polarized transilluminator, which enhances black and white visualization by making necrotic, desiccated areas that are typical of the hypersensitive response appear black.

FIG. **5** shows a tobacco leaf showing that *Erwinia chrysanthemi* hrpN mutants do not elicit the hypersensitive response unless complemented with hrpN+ pCPP2174. Bacteria were suspended at a concentration of 5×10^8 cells/ml in 5 mM MES, pH 6.5, and infiltrated into a tobacco leaf. The leaf was photographed 24 hr later using cross-polarized transillumination as in FIG. **4**. Panels and strains: 1, *E. chrysanthemi* CUCPB5006ΔpelABCE); 2, *E. chrysanthemi* CUCPB5045 (ΔpelABCE $hrpN_{Ech}$546: :Tn5-gusA1); 3, *E. chrysanthemi* CUCPB5045(pCPP2174); 4, buffer alone; 5, *E. chrysanthemi* CUCPB5046 (ΔpelABCE $hrpN_{Ech}$439: Tn5-gusA1); 6, *E. chrysanthemi* CUCPB5046(pCPP2174).

FIG. **6** shows Saintpaulia leaves with rapid necrosis elicited by $HrpN_{Ech}$ and $HrpN_{Ech}$* Pel-deficient *E. chrysanthemi* strains. Leaves were inoculated with bacteria at a concentration of 3×10^8 per milliliter in 5 mM MES, pH 6.5, or purified $HrpN_{Ech}$ at a concentration of 336 μg/ml and photographed 24 hr later as in FIG. **4**. Buffer controls elicited no visible response (not shown). Leaves and treatments: 1, *E. chrysanthemi* CUCPB5006ΔpelABCE); 2, *E. chrysanthemi* CUCPB5045 (ΔpelABCE $hrpN_{Ech}$546: TnphoA); 3, $HrpN_{Ech}$; 4, (left side), *E. chrysanthemi* CUCPB5045 (ΔpelABCE $hrpN_{Ech}$546: Tn5-gusA1); 4 (right side), *E. chrysanthemi* CUCPB5063 (ΔpelABCE outD: :TnphoA $hrpN_{Ech}$546: :Tn5-gusA1)

FIG. **7** shows a diagram of plasmid pCPP19. Significant features are the mobilization (mob) site for conjugation; the cohesive site of λ (cos); and the partition region (par) for stable inheritance of the plasmid. B, BamHI; E, EcoRI; H, HindIII; N, NotI; P, PstI; S, SalI; Sm, SmaI; T3, bacteriophage T3 promoter; T7, bacteriophage T7 promoter; oriV, origin of replication; sp', spectinomycin resistance; Sm', streptomycin resistance.

DETAILED DESCRIPTION OF THE INVENTION AND DRAWINGS

The present invention relates to an isolated DNA molecule encoding for the hypersensitive response elicitor protein or polypeptide from *Erwinia chrysanthemi*. For example, this DNA molecule can comprise the nucleotide sequence corresponding to SEQ. ID. No. 1 as follows:

```
CGATTTTACC  CGGGTGAACG  TGCTATGACC  GACAGCATCA  CGGTATTCGA  CACCGTTACG      60

GCGTTTATGG  CCGCGATGAA  CCGGCATCAG  GCGGCGGCGT  GCTCGCCGCA  ATCCGGCGTC     120

GATCTGGTAT  TTCAGTTTGG  GGACACCGGG  CGTGAACTCA  TGATGCAGAT  TCAGCCGGGG     180

CAGCAATATC  CCGGCATGTT  GCGCACGCTG  CTCGCTCGTC  GTTATCAGCA  GGCGGCAGAG     240

TGCGATGGCT  GCCATCTGTG  CCTGAACGGC  AGCGATGTAT  TGATCCTCTG  GTGGCCGCTG     300
```

-continued

CCGTCGGATC	CCGGCAGTTA	TCCGCAGGTG	ATCGAACGTT	TGTTTGAACT	GGCGGGAATG	360
ACGTTGCCGT	CGCTATCCAT	AGCACCGACG	GCGCGTCCGC	AGACAGGGAA	CGGACGCGCC	420
CGATCATTAA	GATAAAGGCG	GCTTTTTTTA	TTGCAAAACG	GTAACGGTGA	GGAACCGTTT	480
CACCGTCGGC	GTCACTCAGT	AACAAGTATC	CATCATGATG	CCTACATCGG	GATCGGCGTG	540
GGCATCCGTT	GCAGATACTT	TTGCGAACAC	CTGACATGAA	TGAGGAAACG	AAATTATGCA	600
AATTACGATC	AAAGCGCACA	TCGGCGGTGA	TTTGGGCGTC	TCCCGTCTGG	GGCTGCGTGC	660
TCAGGGACTG	AAAGGACTGA	ATTCCGCGGC	TTCATCGCTG	GGTTCCAGCG	TGGATAAACT	720
GAGCAGCACC	ATCGATAAGT	TGACCTCCGC	GCTGACTTCG	ATGATGTTTG	GCGGCGCGCT	780
GGCGCAGGGG	CTGGGCGCCA	GCTCGAAGGG	GCTGGGGATG	AGCAATCAAC	TGGGCCAGTC	840
TTTCGGCAAT	GGCGCGCAGG	GTGCGAGCAA	CCTGCTATCC	GTACCGAAAT	CCGGCGGCGA	900
TGCGTTGTCA	AAAATGTTTG	ATAAAGCGCT	GGACGATCTG	CTGGGTCATG	ACACCGTGAC	960
CAAGCTGACT	AACCAGAGCA	ACCAACTGGC	TAATTCAATG	CTGAACGCCA	GCCAGATGAC	1020
CCAGGGTAAT	ATGAATGCGT	TCGGCAGCGG	TGTGAACAAC	GCACTGTCGT	CCATTCTCGG	1080
CAACGGTCTC	GGCCAGTCGA	TGAGTGGCTT	CTCTCAGCCT	TCTCTGGGGG	CAGGCGGCTT	1140
GCAGGGCCTG	AGCGGCGCGG	GTGCATTCAA	CCAGTTGGGT	AATGCCATCG	GCATGGGCGT	1200
GGGGCAGAAT	GCTGCGCTGA	GTGCGTTGAG	TAACGTCAGC	ACCCACGTAG	ACGGTAACAA	1260
CCGCCACTTT	GTAGATAAAG	AAGATCGCGG	CATGGCGAAA	GAGATCGGCC	AGTTTATGGA	1320
TCAGTATCCG	GAAATATTCG	GTAAACCGGA	ATACCAGAAA	GATGGCTGGA	GTTCGCCGAA	1380
GACGGACGAC	AAATCCTGGG	CTAAAGCGCT	GAGTAAACCG	GATGATACCG	GTATTGACCT	1440
CGCCAGCATG	GACAAATTCC	GTCAGGCGAT	GGGTATGATC	AAAAGCGCGG	TGGCGGGTGA	1500
TACCGGCAAT	ACCAACCTGA	ACCTGCGTGG	CGCGGGCGGT	GCATCGCTGG	GTATCGATGC	1560
GGCTGTCGTC	GGCGATAAAA	TAGCCAACAT	GTCGCTGGGT	AAGCTGGCCA	ACGCCTGATA	1620
ATCTGTGCTG	GCCTGATAAA	GCGGAAACGA	AAAAAGAGAC	GGGGAAGCCT	GTCTCTTTTC	1680
TTATTATGCG	GTTTATGCGG	TTACCTGGAC	CGGTTAATCA	TCGTCATCGA	TCTGGTACAA	1740
ACGCACATTT	TCCCGTTCAT	TCGCGTCGTT	ACGCGCCACA	ATCGCGATGG	CATCTTCCTC	1800
GTCGCTCAGA	TTGCGCCGCT	GATGGGGAAC	GCCGGGTGGA	ATATAGAGAA	ACTCGCCGGC	1860
CAGATGGAGA	CACGTCTGCG	ATAAATCTGT	GCCGTAACGT	GTTTCTATCC	GCCCCTTTAG	1920
CAGATAGATT	GCGGTTTCGT	AATCAACATG	GTAATGCGGT	TCCGCCTGTG	CGCCGGCCGG	1980

-continued

GATCACCACA ATATTCATAG AAAGCTGTCT TGCACCTACC GTATCGCGGG AGATACCGAC 2040

AAAATAGGGC AGTTTTTGCG TGGTATCCGT GGGGTGTTCC GGCCTGACAA TCTTGAGTTG 2100

GTTCGTCATC ATCTTTCTCC ATCTGGGCGA CCTGATCGGT T 2141

Also encompassed by the present invention are fragments of the DNA molecule comprising the nucleotide sequence corresponding to SEQ. ID. No. 1. Suitable fragments capable of eliciting the hypersensitive response (i.e. eliciting necrosis in plants) are constructed by using appropriate restriction sites, revealed by inspection of the DNA molecule's sequence, to: (i) insert an interposon (Fellcy, et al., "Interposon Mutagenesis of Soil and Water Bacteria: a Family of DNA Fragments Designed for in vitro Insertion Mutagenesis of Gram-negative Bacteria, *Gene* 52:147–15 (1987), which is hereby incorporated by reference) such that truncated forms of the hypersensitive response elicitor polypeptide or protein, that lack various amounts of the C-terminus, can be produced or (ii) delete various internal portions of the protein. Alternatively, the sequence can be used to amplify any portion of the coding region, such that it can be cloned into a vector supplying both transcription and translation start signals.

Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide.

The DNA molecule, corresponding to SEQ. ID. No. 1, contains an open reading frame which codes for the hypersensitive response elicitor polypeptide or protein from *Erwinia chrysanthemi* and corresponds to SEQ. ID. No. 2 as follows:

Met 1	Gln	Ile	Thr	Ile 5	Lys	Ala	His	Ile	Gly 10	Gly	Asp	Leu	Gly	Val 15	Ser
Gly	Leu	Gly	Ala 20	Gln	Gly	Leu	Lys	Gly 25	Leu	Asn	Ser	Ala	Ala 30	Ser	Ser
Leu	Gly	Ser 35	Ser	Val	Asp	Lys	Leu 40	Ser	Ser	Thr	Ile	Asp 45	Lys	Leu	Thr
Ser	Ala 50	Leu	Thr	Ser	Met	Met 55	Phe	Gly	Gly	Ala	Leu 60	Ala	Gln	Gly	Leu
Gly 65	Ala	Ser	Ser	Lys	Gly 70	Leu	Gly	Met	Ser	Asn 75	Gln	Leu	Gly	Gln	Ser 80
Phe	Gly	Asn	Gly	Ala 85	Gln	Gly	Ala	Ser	Asn 90	Leu	Leu	Ser	Val	Pro 95	Lys
Ser	Gly	Gly	Asp 100	Ala	Leu	Ser	Lys	Met 105	Phe	Asp	Lys	Ala	Leu 110	Asp	Asp
Leu	Leu	Gly 115	His	Asp	Thr	Val	Thr 120	Lys	Leu	Thr	Asn	Gln 125	Ser	Asn	Gln
Leu	Ala 130	Asn	Ser	Met	Leu	Asn 135	Ala	Ser	Gln	Met	Thr 140	Gln	Gly	Asn	Met
Asn 145	Ala	Phe	Gly	Ser	Gly 150	Val	Asn	Asn	Ala	Leu 155	Ser	Ser	Ile	Leu	Gly 160
Asn	Gly	Leu	Gly	Gln 165	Ser	Met	Ser	Gly	Phe 170	Ser	Gln	Pro	Ser	Leu 175	Gly
Ala	Gly	Gly	Leu 180	Gln	Gly	Leu	Ser	Gly 185	Ala	Gly	Ala	Phe	Asn 190	Gln	Leu
Gly	Asn	Ala 195	Ile	Gly	Met	Gly	Val 200	Gly	Gln	Asn	Ala	Ala 205	Leu	Ser	Ala
Leu	Ser 210	Asn	Val	Ser	Thr	His 215	Val	Asp	Gly	Asn	Asn 220	Arg	His	Phe	Val
Asp 225	Lys	Glu	Asp	Arg	Gly 230	Met	Ala	Lys	Glu	Ile 235	Gly	Gln	Phe	Met	Asp 240
Gln	Tyr	Pro	Glu	Ile 245	Phe	Gly	Lys	Pro	Glu 250	Tyr	Gln	Lys	Asp	Gly 255	Trp

-continued

Ser	Ser	Pro	Lys 260	Thr	Asp	Asp	Lys	Ser 265	Trp	Ala	Lys	Ala	Leu 270	Ser	Lys
Pro	Asp	Asp 275	Asp	Gly	Met	Thr	Gly 280	Ala	Ser	Met	Asp	Lys 285	Phe	Arg	Gln
Ala	Met 290	Gly	Met	Ile	Lys	Ser 295	Ala	Val	Ala	Gly	Asp 300	Thr	Gly	Asn	Thr
Asn 305	Leu	Asn	Leu	Arg	Gly 310	Ala	Gly	Gly	Ala	Ser 315	Leu	Gly	Ile	Asp	Ala 320
Ala	Val	Val	Gly	Asp 325	Lys	Ile	Ala	Asn	Met 330	Ser	Leu	Gly	Lys	Leu 335	Ala
Asn	Ala														

This protein or polypeptide has a molecular weight of 32 to 36 kDa, preferably 34 kDa. It is heat stable (i.e., activity is retained upon boiling for 10 min.), has a glycine content of greater than 16%, and contains no cysteine.

The DNA molecule containing just the open reading frame coding for SEQ. ID. No. 2 has the nucleotide sequence corresponding to SEQ. ID. NO. 6 as follows:

1	ATGCAAATTACGATCAAAGCGCACATCGGCGGTGATTTGGGCGTCTCCGGTCTGGGCCTG	60
61	GGTGCTCAGGGACTGAAAGGACTGAATTCCGCGCTTCATCGCTGGGTTCCAGCGTGGAT	120
121	AAACTGAGCAGCACCATCGATAAGTTGACCTCCGCGCTGACTTCGATGATGTTTGGCGGC	180
181	GCGCTGGCGCAGGGGCTGGGCGCCAGCTCGAAGGGGCTGGGGATGAGCAATCAACTGGGC	240
241	CAGTCTTTCGGCAATGGCGCGCAGGGTGCGAGCAACCTGCTATCCGTACCGAAATCCGGC	300
301	GGCGATGCGTTTGTCAAAAATGTTTTGATAAAGCGCTGGACGATCTGCTGGGTCATGACACC	360
361	GTGACCAAGCTGACTAACCAGAGCAACCAACTGGCTAATTCAATGCTGAACGCCAGCCAG	420
421	ATGACCCAGGGTAATATGAATGCGTTCGGCAGCGGTGTGAACAACGCACTGTCGTCCATT	480
481	CTCGGCAACGGTCTCGGCCAGTCGATGAGTGGCTTCTCTCAGCCTTCTCTGGGGGCAGGC	540
541	GGCTTGCAGGGCCTGAGCGGCGCGGGTGCATTCAACCAGTTGGGTAATGCCATCGGCATG	600
601	GGCGTGGGCAGAATGCTGCTGTGAGTGCTTTGAGTAACGTCAGCACCCACGTAGACGGT	660
661	AACAACCGCCACTTTGTAGATAAAGAAGATCTCGGCATGGCGAAAGAGATCGGCCAGTTT	720
721	ATGGATCAGTATCCGGAAATATTCGGTAAACCGGAATACCAGAAAGATGGCTGGAGTTCG	780
781	CCGAAGACGGACGACAAATCCTGGGCTAAAGCGCTGAGTAAACCGGATGATGACGCTATG	840
841	ACCGGCGCCAGCATGGACAAATTCGGTCAGGCGATGGGTATGATCAAAAGCGCGGTGGCG	900
901	GGTGATACCGGCAATACCAACCTGAACCTGCGTGGCGCGGGCGGTGCATCGCTGGGTATC	960
961	GATGCGGCTGTCGTCGGCGATAAAATAGCCAACATGTCGCTGGGTAAGCTGGCCAACGCC	1020
1021	TGA	1023

The protein or polypeptide of the present invention is preferably produced in purified form (preferably, at least about 80%, more preferably 90% pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is isolated from *E. coli* by lysing and sonication. After washing, the lysate pellet is resuspended in buffer containing Tris-HCl. During dialysis, a precipitate forms from this protein solution. The solution is centrifuged, and the pellet is washed and resuspended in the buffer containing Tris-HCl. Proteins are resolved by electrophoresis through an SDS 12% polyacrylamide gel.

The DNA molecule C encoding the hypersensitive response elicitor polypeptide from *Erwinia chrysanthemi* can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Pat. No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccinia virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK ± or KS ± (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La. Jolla, Calif., which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F. W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," *Gene Expression Technology* vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Laboratory, Cold Springs Harbor, N.Y. (1982), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria or transformed via particle bombardment (i.e. biolistics). The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from those of procaryotic promoters. Furthermore, eucaryotic promotors and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promotors are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, *Methods in Enzymology*, 68:473 (1979), which is hereby incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, lac promotor, trp promotor, recA promotor, ribosomal RNA promotor, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to lacUV5, ompF, bla, lpp, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid trp-lacUV5 (tac) promotor or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operons, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the lac operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as trp, pro, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires a Shine-Dalgarno (SD) sequence about 7–9 bases 5' to the initiation codon (ATG) to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the cro gene or the N gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C,

B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding the hypersensitive response elicitor protein or polypeptide from *Erwinia chrysanthemi* has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

One aspect of the present invention involves using the DNA molecule encoding the hypersensitive response elicitor from *Erwinia chrysanthemi* to transform plants in order to impart localized resistance to pathogens, such as bacteria, fungi, nematodes, and viruses, using a promoter specifically activated by one or more of these pathogens. As a result, when the transgenic plant is infiltrated by such pathogens, the leaves of the plant will undergo localized collapse. This confines the pathogen so that further growth is prevented, and, ultimately, the pathogen perishes. Transformation of plants with the DNA molecule of the present invention is particularly useful where the plant does not exhibit a hypersensitive response to pathogens or is weakly responsive to such pathogens. This requires that $hrpN_{Ea}$ be hooked up to the promotor of a plant gene that the pathogen induces such as PAL_a, CHS, etc. Otherwise, hrpN will kill the plant. It may also be appropriate to transform plants with this DNA molecule where the pathogens they are susceptible to do not elicit a hypersensitive response or elicit a response which is weak.

This aspect of the present invention requires that the subject DNA molecule be incorporated in plants with a suitable promoter which is activated by pathogen infection. Suitable promoters for these purposes include those from the following genes: genes expressed in response to fungus and bacterial infection (e.g., genes encoding phenylalanine ammonia lyase and chalcone synthase) and genes involved in the development of senescence.

The isolated DNA molecule of the present invention can be utilized to impart localized resistance to pathogens for a wide variety of plants, including both monocots and dicots. Although the gene can be inserted into any plant falling within these broad classes, it is particularly useful in crop plants, such as rice, wheat, barley, rye, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum, and sugarcane. The present invention may also be used in conjunction with non-crop plants, including *Arabidopsis thaliana, saintpaulia, petunia, pelargonium,* and *zinnia.*

The expression system of the present invention can also be used to transform virtually any plant cell under suitable conditions. Cells transformed in accordance with the present invention can be grown in vitro in a suitable medium to impart localized resistance to pathogens. Transformed cells can be regenerated into whole plants such that this protein imparts localized resistance to pathogens to the intact transgenic plants. In either case, the plant cells transformed with the recombinant DNA expression system of the present invention are grown and caused to express that DNA in the cells in response to a pathogen to impart localized resistance to pathogens on them.

One technique of transforming plants with the DNA molecule in accordance with the present invention is by contacting the tissue of such plants with an inoculum of a bacteria transformed with a vector comprising a gene in accordance with the present invention which imparts localized resistance to pathogens. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25°–28° C.

Bacteria from the genus Agrobacterium can be utilized to transform plant cells. Suitable species of such bacterium include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes. Agrobacterium tumefaciens* (e.g., strains LBA4404 or EHA105) is particularly useful due to its well-known ability to transform plants.

Another approach to transforming plant cells with a gene which imparts localized resistance to pathogens is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Pat. Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

The hypersensitive response elicitor polypeptide or protein of the present invention can be applied to all or part of the plants listed previously to impart resistance to the plants. In this procedure, the polypeptide or protein alone, mixed with a carrier, and/or with plant treating agents (e.g., fertilizers, insecticides, fungicides, etc.) can be applied to plant. Such application requires infiltration of the polypeptide or protein into the plant by, for example, injection, leaf abrasion proximate to the time of application, or high pressure spraying.

The isolated DNA molecule of the present invention could be used to generate male sterile plants used in plant breeding for generation of hybrids. A promoter from a plant gene involved specifically in pollen development could be used to express the hypersensitive response elicitor polypeptide or protein in anthers, resulting in death of the anther and/or pollen.

The hypersensitive response elicitor polypeptide or protein of the present invention could be used as a regulatable suicide factor to kill a transgenic plant on demand. If it were engineered to be expressed from a promoter that is induced in response to a normally nontoxic, environmentally friendly, inducer molecule, the plant could be killed by spraying with the inducer molecule rather than with herbicides. This could be used, for example, instead of herbicides to kill potato vines to facilitate harvest and reduce the devastating tuber blight phase of late blight. This could also be linked with other transgenes to permit the control of a transgenic plant that escaped or outcrossed with a weed. For example, a concern with pathogen-derived resistance to viruses is that the virus resistance genes could spread from the transgenic crop to related weeds that are normally held in check by natural virus infections. These new, more hardy weeds, would now be sensitive to killing by the inducer of the gene.

The hypersensitive response elicitor polypeptide or protein of the present invention may be used as a selective herbicide in synergistic combination with an avirulence protein that interacts with a resistance gene product that is unique to the targeted weed (or is lacking from crop plants).

It may be possible to use the hypersensitive response elicitor polypeptide or protein of the present invention in a tissue culture selection scheme to select cultures that are resistant to it. Regenerated plants may then be resistant to *E. chrysanthermi* and may even be resistant to a wide range of plant pathogenic bacteria. It is possible that this protein or polypeptide has effects on animal cells that could be exploited for medical use, insect control.

The hypersensitive response elicitor protein or polypeptide can also be used to raise monoclonal or polyclonal antibodies by conventional procedures. At least the binding portions of these antibodies can be sequenced and encoding DNA synthesized. The encoding DNA molecule can be used to transform plants together with a promoter which causes expression of the encoded protein when the plant is infected by *Erwinia chrysanthemi*. The expressed protein will bind to the pathogen and help prevent the usual necrotic response. Antibodies to this protein or polypeptide of the present invention could also be used to identify pathogenic Erwinia.

EXAMPLE 1

Bacterial Strains, Plasmids and Culture Conditions

Bacterial strains and plasmids are listed in Table 1.

TABLE 1

Bacterial strains and plasmids utilized

Designation	Relevant characteristics[a]	Reference or source
Escherichia coli		
ED8767	supE44 supF58 lacIS3;r_K^- m_{K+}) recA56 galK2 galT22 metB1	(Sambrook et al. 1989)[b]
DH5α	supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 Nal[r]	(Hanahan 1983)[c]; Life Technologies, Inc. (Grand Island, NY)
DH12B	recA Δ(mrr-hsRMS-mcrBC) ϕ80 lacZΔM15 ΔlacX74 desR recA1 endA1 araD139 Δ(ara, leu) 7697 galU galK rpsL nupG	(Grant et al. 1990)[d]; Life Technologies, Inc.
Erwinia chrysanthemi		
EC16	Wild-type strain	(Burkholder et al. 1953)[e]
AC4150	Spontaneous Nal[r] derivative of EC16	(Chatterjee et al. 1983)[f]
CUCPB5006	ΔpelB pelC);:28bp Δ(pelA pelE) derivative of AC4150	(He and Collmer 1990)[g]
CUCPB5030	ouID::TnphoA derivative of CUCPB5006	(Bauer et al. 1994)[h]
CUCPB5045	$hrpN_{Ech}$546::Tn5-guaA1 derivative of CUCPB5006	Described in this application
CUCPB5046	$hrpN_{Ech}$539::Tn5-guaA1 derivative of CUCPB5006	Described in this application
CUCPB5063	$hrpN_{Ech}$546::Tn5-guaA1 derivative of CUCPB5030	Described in this application
CUCPB5049	$hrpN_{Ech}$539::Tn5-guaA1 derivative of AC4150	Described in this application
Erwinia amylovora		
Ea321	Wild type	ATCC 49947;

TABLE 1-continued

Bacterial strains and plasmids utilized

Designation	Relevant characteristics[a]	Reference or source
		UNPB 1367
Ea321TS	$hrpN_{Ea}$::Tn5tsc1 derivative of Ea321	(Wei et al. 1992)[i]
Plasmids and phage		
pBluescript II SK(−)	Amp^r	Stratagene (La Jolla CA)
pCPP19	Cosmid Vector, Sp^r/Sm^r	See FIG. 7.
pUC119	Amp^r plasmid vector	(Vieira and Messing 1987)[j]
pSE280	Amp^r plasmid vector with superpolylinker downstream of tac promoter	(Brosius 1989)[k]
pCPP2030	pCPP19 carrying *E. chrysanthemi* DNA hybridizing with *E. amylovora* hrp genes in pCPP1033	(Bauer et al. 1994)[h]
pCPP1084	pBluescipt M13+ carrying $hrpN_{Ea}$, on 1.3 kb HindIII fragment	(Wei et al. 1992)[i]
pCPP2157	pCPP19 carrying *E. chrysanthemi* DNA hybridizing with *E. amylovora* hrpN	Described in this application
pCPP2142	8.3 kb SalI subclone from pCPP2157 in pUC119	Described in this application
pCPP2141	3.1 kb PstI subclone from pCPP2157 in pBluescipt II SK(−) $hrpN_{Ech}$ in opposite orientation from vector lac promoter	Described in this application
pCPP2172	3.1 kb PstI subclone from pCPP2157 in pBluescipt II SK(−) $hrpN_{Ech}$ in same orientation as vector lac promoter	Described in this application
pCPP2174	1.0 kb $hrpN_{Ech}$ PCR product cloned in NcoI-HindIII sites of pSE280	Described in this application
λ::Tn5-gusA1	Tn5 derivative for generating transcriptional fusions with uidA reporter; Kan^r, Tet^r	(Sharma and Signer 1990)[l]

[a] Amp^r = ampicillin resistance; Nal^r = nalidixic acid resistance; Sm^r = streptomycin resistance; Sp^r = spectinomycin resistance; Tet^r = tetracycline resistance

[b] Sambrook, J., Fritsch, E. F., and Maniatis, T., Molecular Cloning. A laboratory Manual, Second Edition. Cold Spring Harbor, Cold Spring Harbor (1989), which is hereby incorporated by reference.

[c] Hanahan, D., "Studies on Transformation of *Escherichia coli* with Plasmids," J. Mol. Biol. 166:557–580 (1983), which is hereby incorporated by reference.

[d] Grant, S. G. N., Jessee, J., Bloom, F. R., and Hanahan, D., "Differential Plasmid. Rescue from Transgenic Mouse DNAs in *Escherichia coli* Methylation-restriction Mutants," Proc. Nat. Acad. Sci. U.S.A. 87:4645–46949 (1990), which is hereby incorporated by reference.

[e] Burkholder, W. H., McFadden, L. A., and Dimock, A. W., "A Bacterial Blight of Chrysanthemums," Phytopathology 43:522–526 (1953), which is hereby incorporated by reference.

[f] Chatterjee, A. K., Thurn, K. K., and Fesse, D. A. "Tn5 Induced Mutations in the Enterobacterial Phytopathogen *Erwinia chrysanthemi*," Appl. Environ. Microbiol. 45:644–650 (1983), which is hereby incorporated by reference.

TABLE 1-continued

Designation	Relevant characteristics	Reference or source
Bacterial strains and plasmids utilized		

[1]He, S. Y., and Collmer, A., "Molecular Cloning, Nucleotide Sequences and Marker-exchange Mutagenesis of the Exo-poly-α-D-galactosonuidase-encoding pehX Gene of *Erwinia chrysanthemi* EC16," J. Bacteriol. 172:4088–4095 (1990), which is hereby incorporated by reference.
[2]Bauer, D. W., Bogdanove, A. J., Beer, S. V., and Collmer, A., "*Erwinia Chrysanthemi* hrp Genes and their Involvement in Soft Rot Pathogenesis and Elicitation of the Hypersensitive Response," Mol Plant-Microbe Interact., 7:573–581 (1994), which is hereby incorporated by reference.
[3]Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85–88 (1992), which is hereby incorporated by reference.
[4]Vieira, J., and Messing, J., "Production of Single-stranded Plasmid DNA," Meth. Enzymol. 153:3–11 (1987), which is hereby incorporated by reference.
[5]Brosius, J., "Superpolylinkers in Cloning and Expression Vectors," DNA 8:759–777 (1989), which is hereby incorporated by reference.
[6]Sharma, S. B., and Signer, E. R., "Temporal and Spatial Regulation of the Symbiotic Genes of *Rhizobium meliloti* in Planta Revealed by Transposon Tn5-gusA," Genes Develop. 4:344–356 (1990), which is hereby incorporated by reference.

E. chrysanthemi was routinely grown in King's medium B ("KB") (King, E. O. et al., "Two Simple Media for the Demonstration of Pyocyanin and Fluorescein," *J. Lab. Med.* 22:301–307 (1954), which is hereby incorporated by reference) at 30° C., *E. coli* in LM medium (Hanahan, D., "Studies on transformation of *Escherichia coli* with Plasmids," *J. Mol. Biol.* 166:557–580 (1983), which is incorporated by reference) at 37° C., and *E. amylovora* in LB medium at 28°–30° C. The following antibiotics were used in selective media in the amounts indicated (μg/ml), except where noted: ampicillin, 100; kanamycin, 50; spectinomycin, 50; and streptomycin, 25.

EXAMPLE 2

General DNA Manipulations

Plasmid DNA manipulations, colony blotting, and Southern blot analyses were performed using standard techniques (Sambrook, J., et al., "Molecular Cloning. A Laboratory Manual," Second Edition, Cold Spring Harbor, Cold Spring Harbor (1989), which is incorporated by reference). Deletions for sequencing were constructed with the Erase-a-Base Kit (Promega, Madison, Wis.). Double stranded DNA sequencing templates were prepared using Qiagen Plasmid Mini Kits (Chatsworth, Calif). Sequencing was performed using the Sequenase Version 2 kit (U.S. Biochemical, Cleveland, Ohio). The Tn5-gusAl insertion points were determined on an Applied Biosystems (Foster City, Calif.) Automated DNA Sequencer Model 373A by the Cornell Biotechnology Center. DNA sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux, J., et al., "A Comprehensive Set of Sequence Analysis Programs for the VAX," *Gene* 12:387–395 (1984), which is hereby incorporated by reference) Comparison of $HrpN_{Ech}$ (i e. the gene encoding the hypersensitive response elicitor polypeptide or protein from *Erwinia chrysanthemi*) and $HrpN_{Ea}$ (i.e. the gene encoding the hypersensitive response elicitor polypeptide or protein from *Erwinia amylovora*) by the Gap Program was done with a gap weight of 5.0 and a gap length weight of 0.3. Marker-exchange mutagenesis was performed as described (Bauer, D. W., et al., "*Erwinia chrysanthemi* hrp Genes and Their Involvement in Soft Rot Pathogenesis and Elicitation of the Hypersensitive Response," *Mol. Plant-Microbe Interact.* 7:573–581 (1994), which is incorporated by reference). The oligonucleotide used to determine the location of Tn5-gusAl insertions in $hrpN_{ECh}$ was TGACCTG-CAGCCAAGCTTTCC (SEQ. ID. No. 3). The oligonucleotide used as the first primer to amplify the $hrpN_{Ech}$ ORF and to introduce a NcoI site at the 5' end of the gene was AGTACCATGGTTATTACGATCAAAGCGCAC (SEQ. ID. No. 4); the one used as the second primer to introduce an XhoI site at the 3' end of the gene was AGATCTC-GAGGGCGTTGGCCAGCTTACC (SEQ. ID. No. 5). Primers were synthesized by Integrated DNA Technologies (Coralville, Iowa).

EXAMPLE 3

Protein Manipulations

$HrpN_{Ech}$ was purified from *E. coli* Dh5α(pCPP2172) cultures grown at 30° C. to stationary phase in 50 ml of Terrific Broth (Sambrook, J., et al., "Molecular Cloning. A Laboratory Manual," Second Edition, Cold Spring Harbor, Cold Spring Harbor (1989), which is incorporated by reference) supplemented with ampicillin at a concentration of 200 μg/ml. Cells were lysed by lysozyme treatment and sonication as described (Sambrook J., et al., "Molecular Cloning. A Laboratory Manual," Second Edition, *Cold Spring Harbor*, Cold Spring Harbor (1989), which is incorporated by reference). The lysate pellet was washed twice with 9 volumes of lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and reharvested each time by centrifugation at 12,000xg for 15 min. The pellet was resuspended in 2.0 ml lysis buffer containing 0.1 mM PMSF, dissolved by the addition of 2.5 ml of 8M guanidine-HCl in lysis buffer and then diluted with 5.0 ml water. The protein solution was dialyzed in SpectraPor #1 dialysis tubing against 2.0 l of 5 mM MES, pH 6.5, containing 0.05 mM PMSF. The precipitate that formed during dialysis and the solution were centrifuged for 15 min at 4,300xg. The pellet was washed once with 10 ml of 5 mM MES, pH. 6.5, with 0.1 mM PMSF and then resuspended in 2.0 ml of the same buffer. Protein concentrations of homogeneous suspensions were determined following dissolution in the reagents of the dye-binding assay of Bradford (Bradford, M., "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye-binding," *Anal. Biochem*, 92:248–2254 (1976), which is incorporated by reference). Proteins in crude cell lysates or following purification were resolved by electrophoresis through an SDS 12% polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue R. The N-terminal sequence of purified $HrpN_{Ech}$ was determined at the Cornell University Biotechnology Program Analysis Facility.

EXAMPLE 4

Plant Assays

Tobacco (*Nicotiana tabacum* L. var Xanthi), tomato (Lycopersicon esculentum Mill. var Sweet 199), pepper (*Capsicum annuum* L. var *Sweet Hungarian*), Saintpaulia (*S. ionantha Wendl.*var. Paris), *petunia* (*P. grandiflora Juss.* var. Blue Frost), pelargonium (P. hortorum Bailey), winter squash (*Cucurbita maxima* Duchesne.), and Zinnia (Z. elegans Jacq.) plants were grown under greenhouse conditions or purchased at a local garden shop and then maintained in the laboratory at room temperature, with incident daylight supplemented with a 500 W halogen lamp, for hypersensitive response assays. Witloof chicory (*Cichorium intybus L.*) "Belgian endive" heads were purchased from a local supermarket. Bacterial inoculum was prepared and delivered as previously described (Bauer, D. W., et al., "*Erwinia chrysanthemi* hrp Genes and Their Involvement in Soft Rot Pathogenesis and Elicitation of the Hypersensitive

Response," *Mol. Plant-Microbe Interact,* 7:573–581 (1994), which is incorporated by reference). Briefly, to assay soft-rot pathogenesis, 5 μl of inoculum was applied to a small wound in detached chicory leaves, to assay for hypersensitive response elicitation, inoculum was injected into the intercellular spaces of plant leaves with a needle-less plastic syringe. Hereafter, in these examples, this injection procedure is referred to as "infiltrated" or as "infiltrations".

EXAMPLE 5

Molecular Cloning of the *E. chrysanthemi* $hrpN_{Ech}$ Gene.

Eighteen cosmids containing *E. chrysanthemi* DNA sequences hybridizing with a region of the *E. amylovora* hrp cluster that is widely conserved in plant pathogenic bacteria were previously isolated (Bauer, D. W., et al., "*Erwinia chrysanthemi* hrp Genes and Their Involvement in Soft Rot Pathogenesis and Elicitation of the Hypersensitive Response," *Mol. Plant-Microbe Interact,* 7:573–581 (1994), which is incorporated by reference). The pattern of restriction fragments released from these cosmids indicated they all contained overlapping inserts from the same region of the *E. chrysanthemi* genome. The cosmids were probed in colony blots with a 1.3 kb HindIII fragment from pCPP1084, which contains the *E. amylovora* hrpN gene (Wei, Z.-M., et al., "Harpin Elicitor of the Hypersensitive Response Produced by the Plant Pathogen," *Science* 257:85–88 (1992), which is incorporated by reference). pCPP2157, one of the three cosmids hybridizing with the probe, was digested with several restriction enzymes, and the location of the $hrpN_{Ech}$ gene in these fragments was determined by probing a Southern blot with the *E. amylovora* HindIII fragment. Two fragments, each containing the entire $hrpN_{Ech}$ gene, were subcloned into different vectors: pCPP2142 contained an 8.3 kb SalI fragment in pUC119 (Vieira, J., et al., "Production of Single-stranded Plasmid DNA, *Meth. Enzymol,* 153:3–11 (1987), which is incorporated by reference), and pCPP2141 contained a 3.1 kb PstI fragment in pBluescript II SK(–) (Stratagene, La Jolla, Calif.) and was used to sequence the $hrpN_{Ech}$ gene.

EXAMPLE 6

Sequence of $hrpN_{Ech}$

The nucleotide sequence of a 2.4 kb region of pCPP2141 encompassing $hrpN_{Ech}$ was determined. The portion of that sequence extending from the putative ribosome binding site through the $hrpN_{Ech}$ coding sequence to a putative rho-independent terminator is presented in FIG. 1. The typical ribosome-binding site, consisting of GAGGA, was located 10 bases upstream of the ATG translational initiation codon. No promoter sequences were discernible upstream of $hrpN_{Ech}$. Instead, the presence of another open reading frame suggested that $hrpN_{Ech}$ is the last open reading frame in a polycistronic operon. $hrpN_{Ech}$ codes for a predicted protein that is 34.3 kD, rich in glycine (16.2%) and lacking in cysteine. Comparison of the amino acid sequences of the predicted $hrpN_{Ea}$ and $hrpN_{Ech}$ products revealed extensive similarity, particularly in the C-terminal halves of the proteins (FIG. **2**). The overall identity of the hrpN genes and proteins was 66.9% and 45.5%, as determined by the FASTA and Gap algorithms, respectively (Devereaux, J., et al., "A Comprehensive Set of Sequence Analysis Programs for the VAX," *Gene* 12:387–395 (1984); Pearson, W. R., et al., "Improved Tools for Biological Sequence Comparison," *Proc. Natl. Acad. Sci. U.S.A.,* 85:2444–2448 (1988), which are hereby incorporated by reference).

The direction of $hrpN_{Ech}$ transcription, the size of the predicted product, and the translation start site were confirmed by reclening the 3.1 kb PstI fragment from pCPP2157 and selecting a clone with the fragment in pBluescript II SK(–) in the opposite orientation from pCPP2141 to produce pCPP2172. *E. coli* DH5α(pCPP2172) expressed $hrpN_{Ech}$ from the vector lac promoter and produced high levels of a protein with an estimated molecular mass of 36 kD in SDS polyacrylamide gels, which is close to the predicted size (FIG. **3**). Furthermore, the N-terminal 10 amino acids of the 36 kD protein, determined by microsequencing following purification as described below, corresponded with the predicted N-terminus of $HrpN_{Ech}$. No N-terminal signal sequence for targeting to the general export (Sec) pathway was discernible in the $HrpN_{Ech}$ sequence, and the data showed no evidence of processing of the N-terminus.

EXAMPLE 7

Purification of the $hrpN_{Ech}$ Product and Demonstration of its Hypersensitive Response Elicitor Activity in Tobacco

When DH5α(pCPP2172) cells were disrupted by sonication and then centrifuged, most of the $HrpN_{Ech}$ protein sedimented with the cell debris. However, soluble $HrpN_{Ech}$ could be released from this material by treatment with 4.5M guanidine-HCl. This suggested that the protein formed inclusion bodies which could be exploited for purification. As detailed in Example 3, it was found that $HrpN_{Ech}$ renaturated when the guanidine-HCl was removed by dialysis against dilute buffer. The $HrpN_{Ech}$ precipitate could be washed and resuspended in buffer, in which it formed a fine suspension. SDS polyacrylamide gel analysis revealed the suspension to be electrophoretically homogeneous Hrp-N_{Ech} (FIG. **3**).

Cell-free lysates from *E. coli* DH5α(pCPP2172) cells grown in LB medium were infiltrated into tobacco leaves. Necrosis typical of the hypersensitive response developed within 18 hr, whereas leaf panels infiltrated with identically prepared lysates of DH5α(pBluescript SK–) showed no response. The suspension of purified $HrpN_{Ech}$ at a concentration of 336 μg/ml also caused a necrotic response within 18 hrs. that was indistinguishable from that caused by *E. chrysanthemi* CUCBP5030 or cell-free lysates from *E. coli* DH5α(pCPP2172) (FIG. **4**). Tobacco plants vary in their sensitivity to harpins, and elicitation of the hypersensitive response by $HrpN_{Ech}$ at lower concentrations was found to be variable. Consequently, a concentration of 336 μg/ml was used in all subsequent experiments. The concentration of $HrpN_{Ech}$ that is soluble in apoplastic fluids is unknown. To determine the heat stability of $HrpN_{Ech}$, the suspension of purified protein was incubated at 100° C. for 15 min and then infiltrated into a tobacco leaf. There was no apparent diminution in its ability to elicit the hypersensitive response. These observations indicated that $HrpN_{Ech}$ is sufficient to account for the ability of *E. chrysanthemi* to elicit the hypersensitive response in tobacco.

EXAMPLE 8

$hrpN_{Ech}$ Mutants Fail to Elicit the Hypersensitive response in Tobacco

E. coli DH10B(pCPP2142) was mutagenized with Tn5-gusA1 (Sharma, S. B., et al., "Temporal and Spatial Regulation of the Symbiotic Genes of *Rhizobium meliloti* in Planta Revealed by Transposon TN5-gusA, *Genes Develop* 4:344–356 (1990), which is incorporated by reference). Plasmid DNA was isolated from kanamycin-resistant colonies and transformed into *E. coli* DH5α, with selection for kanamycin resistance. Plasmids containing Tn5-gusA1 were analyzed by restriction mapping. Two independent insertions in an 0.82 kb ClaI fragment internal to $hrpN_{Ech}$ were chosen for further study. The precise location and orientation of these insertions was determined by using a primer that

hybridizes to Tn5-gusA1 DNA upstream of gusA to sequence into the disrupted *E. chrysanthemi* DNA (FIG. 1). *E. coli* DH5α(pCPP2142) cells carrying the Tn5-gusA1 insertion at nucleotide 439 of the $hrpN_{Ech}$ open reading frame (with gusA and $hrpN_{Ech}$ in the same orientation) produced dark blue colonies indicative of β-glucuronidase activity on LM agar supplemented with 5-bromo-4-chloro-3-indolyl β-D-glucuronide. Whether gusA was expressed from an *E. chrysanthemi* promoter or the vector lac promoter was not determined. The $hrpN_{Ech}$439: :Tn5-gusA1 and $hrpN_{Ech}$546: :Tn5-gusA1 mutations were marker-exchanged into the genome of *E. chrysanthemi* CUCPB5006 (ΔpelABCE) to produce mutants CUCPB5046 and CUCPB5045, respectively. Neither of the $hrpN_{Ech}$ mutants elicited a visible reaction in tobacco leaves (FIG. 5.)

EXAMPLE 9

E. chrysanthemi $hrpN_{Ech}$ Mutations can be Complemented in trans with $hrpN_{Ech}$ but not with $hrpN_{Ea}$

The presence of a typical rho-independent terminator just downstream of the $hrpN_{Ech}$ open reading frame suggested that mutations in the gene would not have polar effects on any other genes and that the hypersensitive response elicitation phenotype should be restored by a $hrpN_{Ech}$ subclone. Because pCPP2172 carried 2 kb of *E. chrysanthemi* DNA in addition to $hrpN_{Ech}$, a precise subclone of the gene was constructed for this purpose. Oligonucleotides were used to PCR-amplify the $hrpN_{Ech}$ open reading frame and to introduce terminal NcoI and XhoI sites. The introduction of the restriction sites resulted in changing the second residue in the protein from glutamine to valine and adding a leucine and a glutamic acid residue to the C-terminus. The resulting DNA fragment was ligated into XhoI/NcoI-digested pSE280, creating pCPP2174, in which hrp_{Ech} was under control of the vector tac promoter. *E. chrysanthemi* CUCPB5045(pCPP2174) and CUCPB5046(pCPP2174) possessed hypersensitive response elicitor activity (FIG. 5). Hypersensitive response elicitor activity could also be restored to these mutants by pCPP2142 and pCPP2171, but not by pCPP2141. Thus, the production of $HrPN_{Ech}$ is essential for elicitation of the hypersensitive response by *E. chrysanthemi* CUCPB5006.

The feasibility of testing the interchangeability of the hrpN genes of *F. chrysanthemi* and *E. amylovora* was supported by the observation that hypersensitive response elicitation activity could be restored to hrpN mutants in each species (*E. chrysanthemi* CUCPB5045 and *E. amylovora* Ea321T5) by their respective $hrpN^-$ subclones (pCPP2142 and pCPP1084). pCPP2142 was used for this purpose, because preliminary immunoblot experiments indicated that the level of $hrpN_{Ech}$ expression by this plasmid, though relatively high, most closely approximated expression of the native hrpN gene in *E. amylovora*. However, despite good heterologous expression of the hrpN genes, hypersensitive response elicitation activity was not restored in either *E. amylovora* Ea321T5(pCPP2142) or *E. chrysanthemi* (pCPP1084). Thus, the genes do not appear to be functionally interchangeable.

EXAMPLE 10

E. chrysanthemi $hrpN_{Ech}$ Mutants have a Reduced Ability to Incite Lesions in Witloof Chicory.

The $hrpN_{Ech}$439: :Tn5-gusA1 mutation was marker-exchanged into the genome of wild-type strain AC4150. The resulting mutant, CUCPB**5049**, was analyzed for its virulence in witloof chicory. Leaves were inoculated at small wounds with 2×10^8 cells of mutant and wild-type strains, as previously described (Bauer, et al., "*Erwinia chrysanthemi* hrp Genes and Their Involvement in Soft Rot Pathogenesis and Elicitation of the Hypersensitive Response," *Mol. Plant-Microbe Interact*, 7:573–581 (1994), which is incorporated by reference). The level of inoculum corresponded with the experimentally determined ED_{50} of the wild-type strain for the batch of chicory heads used. The approximate surface area of macerated lesions was determined 72 hr after inoculation. The mutations did not abolish the pathogenicity of *E. chrysanthemi*, but significantly reduced the number of successful lesions (Table 2).

TABLE 2

Effects of $hrpN_{Ech}$ mutation on the ability of *Erwinia chrysanthemi* to incite lesions on witloof chicory leaves

Strain	Number of lesions per 20 inoculations[a]	Size of lesions (mm^2; mean ± SD)[b]
AC4150 (wild type)	16	80 ± 55
CUCPB5049 ($hrpz_{Ech}$429::Tn5-gusA1)	8[c]	89 ± 42

[a]Each witloof chicory leaf was inoculated at two equivalent sites with 2×10^8 bacterial cells; one site received the $hrpN_{Ech}$ mutant, the other the parental wild-type strain; lesions were indicated by browning and maceration around the site of inoculation.

[b]Values represent the product of the length and width of the lesion.

[c]Different from the wild-type strain ($P < 0.05$), as determined by the McNemar test (Conover, W. J., "Practical Nonparametric Statistics", 2 ed., John Wiley and Sons, New York (1980), which is incorporated by reference).

EXAMPLE 11

Elicitation of a Rapid Necrosis in Several Plants by *E. chrysanthemi* is Dependent on $HrPN_{Ech}$.

To determine whether *E. chrysanthemi* could cause a $HrpN_{Ech}$-dependent necrosis in plants other than tobacco, a variety of plants were infiltrated with purified $HrpN_{Ech}$ or inoculated with Pel-deficient *E. chrysanthemi* strains. The strains used were CUCPB5006 and its $hrpN_{Ech}$546: :Tn5-gusA1 derivative CUCPB5045 and CUCPB5030 (ΔpelABCE outD: :TnphoA) and its $hrpN_{Ech}$546: :Tn5-gusA1 derivative CUCPB5063. The results with *Saintpaulia ionantha* are shown in FIG. **6** and for all plants are summarized in Table 3.

TABLE 3

Elicitation of necrosis in various plants by $HrpN_{Ech}$ and by *E. chrysanthemi* strains that are deficient in Pel production but not $HrpN_{Ech}$ production

Plant	$HrpN_{Ech}$[a]	CUCPB5006 (ΔpelABCE)[b]	CUCPB5045 (ΔpelABCE $hrpN_{Ech}$546::Tn5-gusA1)	CUCPB5030 (ΔpelABCE outD::TnphoA)	CUCPB5063 (ΔpelABCE outD::TnphoA $hrpN_{Ech}$546::Tn5-gusA1)
Tobacco	+	+	–	+	–
Tomato	+	+	–	+	–

TABLE 3-continued

Elicitation of necrosis in various plants by $HrpN_{Ech}$ and by *E. chrysanthemi* strains that are deficient in Pel production but not $HrpN_{Ech}$ production

Plant	$HrpN_{Ech}$[a]	CUCPB5006 (ApelABCE)[b]	CUCPB5045 (ApelABCE $hrpN_{Ech}$546::Tn5-gusA1)	CUCPB5030 (ApelABCE outD::TnphoA)	CUCPB5063 (ApelABCE outD::TnphoA $hrpN_{Ech}$546::Tn5-gusA1)
Pepper	+	+	–	+	–
Saintpaulia	+	+	–	+	–
Petunia	+	+	–	+	–
Pelargonium	+	+	–	+	–
Squash	–	–	–	–	–
Zinnia	–	–	–	–	–

[a]Leaves on plants were infiltrated with $HrpN_{Ech}$ at a concentration of 336 μg/ml and observed macroscopically 24 hr later for necrosis and collapse of the infiltrated area (+) or absence of any response (–).
[b]Leaves on plants were infiltrated with bacteria at a concentration of 5×10^8 and scored for responses as described above.

They yield several general observations. Plants responded either to both isolated $HrpN_{Ech}$ and $hrpN_{Ech}^-$ bacteria or to neither. Plants that responded to either treatment produced a noamacerated, hypersensitive response-like necrosis that developed between 12 and 24 hr after infiltration. $HrpN_{Ech}^-$ mutants failed to elicit a response elicited in the plants tested, indicating that residual Pel isozymes or other proteins travelling the Out pathway were not involved in producing the hypersensitive response-like necrosis. The results argue that $HrpN_{Ech}$ is the only elicitor of the hypersensitive response produced by *E. chrysanthemi*.

E. chrysanthemi was found to produce a protein with many similarities to the harpin of *E. amylovora*. The two proteins share significant amino acid sequence identity, similar physical properties, and the ability to elicit the hypersensitive response in a variety of plants. Mutations in the $hrpN_{Ech}$ gene indicate that, as with *E. amylovora*, harpin production is required for elicitation of the hypersensitive response. Furthermore, both harpins contribute to bacterial pathogenicity, albeit to different degrees. $HrpN_{Ea}$ is essential for *E. amylovora* to produce symptoms in highly susceptible, immature pear fruit (Wei, et al. "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," *Science* 257:85–88 (1992), which is incorporated by reference), whereas $HrpN_{Ech}$ merely increases the frequency of successful *E. chrysanthemi* infections in susceptible witloof chicory leaves. Nevertheless, the finding that harpins play some role in the pathogenicity of such disparate pathogens suggests that these proteins have a conserved and widespread function in bacterial plant pathogenesis. We will consider below $HrpN_{Ech}$ with regard to the protein secretion pathways, extracellular virulence proteins, and wide host range of *E. chrysanthemi*.

E. chrysanthemi secretes proteins by multiple, independent pathways. Several protease isozymes are secreted by the Sec-independent (ABC-transporter or Type I) pathway; pectic enzymes and cellulase are secreted by the Sec-dependent (general secretion or Type II) pathway; and, $HrpN_{Ech}$ is likely to be secreted by the Sec-independent Hrp (Type III) pathway (Salmond, G. P. C., et al., "Secretion of Extracellular Virulence Factors by Plant Pathogenic Bacteria," *Annu. Rev. Phytopathol.* 32:181–200 (1994), which is incorporated by reference). The expectation that $HrpN_{Ech}$ is secreted by the Hrp pathway is supported by several lines of indirect evidence: (i) Hrp secretion pathway mutants have revealed that other members of this class of glycine-rich, heat-stable elicitor proteins, the *E. amylovora* $HrpN_{Ea}$, *P. syringae* pv. *syringae* HrpZ, and *P. solanacearum* PopA1 proteins, are secreted by this pathway (He, S. Y., et al., "*Pseudomonas syringae* pv. *syringae* $Harpin_{Pss}$: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," (1993); Wei, Z.-M., et al., "Hrpl of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family", *J. Bacteriol.* 175:7958–7967 (1993); Arlat, M., et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," *EMBO J.* 13:543–553 (1994), which are hereby incorporated by reference); (ii) mutation of the *E. chrysanthemi* homolog of an *E. amylovora* gene involved in $HrpN_{Ea}$ secretion abolishes the ability of *E. chrysanthemi* to elicit the hypersensitive response, whereas mutation of the Out (Type II) pathway of *E. chrysanthemi* does not abolish the hypersensitive response; and (iii) $HrpN_{Ech}$ appears to be the only hypersensitive response elicitor produced by *E. chrysanthemi* (as discussed further below), suggesting that the effect of the putative hrp secretion gene mutation is on $HrpN_{Ech}$.

Attempts to demonstrate directly hrp-dependent secretion of $HrpN_{Ech}$ have been thwarted by apparent instability of the protein in *E. chrysanthemi*. Using the cell fractionation and immunoblotting procedures of He, S. Y., et al., "Pseudomonas syringae pv. syringae $Harpin_{Pss}$: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," *Cell* 73:1255–66 (1993), which is hereby incorporated by reference, and polyclonal anti-$HrpN_{Ea}$ antibodies that cross-react with $HrpN_{Ech}$ (Wei, Z.-M., et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," *Science* 257:85–88 (1992), which is incorporated by reference), we have observed the presence of $HrpN_{Ech}$ in the cell-bound fraction of *E. chrysanthemi*. However, some culture preparations unexpectedly lack the protein, and no preparations reveal accumulation of the protein in the culture supernatant fraction. It is possible that $HrpN_{Ech}$ aggregates upon secretion and, therefore, precipitates from the medium. It is interesting that several of the Yersinia spp. Yop virulence proteins aggregate in the medium upon secretion via the Type III pathway (Michiels, T., et al., "Secretion of Yop Proteins by Yersiniae," *Infect. Immun.* 58:2840–2849 (1990), which is incorporated by reference). Similarly, $HrpN_{Ea}$ has a propensity to form aggregates or to associate with an insoluble membrane fraction (Wei, Z.-M, et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia*

amylovora," Science 257:85–88 (1992), which is incorporated by reference).

It is significant that there is little difference in the plant interaction phenotypes of *E. chrysanthemi* mutants deficient in either $HrpN_{Ech}$ or a putative component of the Hrp secretion pathway (Bauer, D. W., et al., *"Erwinia chrysanthemi* hrp Genes and Their Involvement in Soft Rot Pathogenesis and Elicitation of the Hypersensitive Response," *Mol. Plant-Microbe Interact,* 7:573–581 (1994), which is incorporated by reference). Both mutations abolish the ability of Pel-deficient strains to elicit the hypersensitive response, and they both reduce the frequency of successful infectious incited by fully pectolytic strains in witloof chicory leaves without affecting the size of the macerated lesions that do develop. This pattern contrasts with that observed with mutations affecting Pel isozymes and the Out pathway. Maceration virulence is merely reduced by individual pel mutations, whereas it is abolished by out mutations. This is because multiple Pel isozymes (and possibly other enzymes) contribute quantitatively to virulence, but all of the Pel isozymes appear to be dependent on the Out pathway for secretion from the bacterial cell. The simplest interpretation of the observations with *E. chrysanthemi* hrp mutants is that $HrpN_{Ech}$ is the only protein travelling the Hrp pathway that has a detectable effect on the interaction of *E. chrysanthemi* EC16 with the plants tested.

The primacy of $HrpN_{Ech}$ in the *E. chrysanthemi* Hrp system is further supported by the observations that $hrpN_{Ech}$ mutants failed to elicit necrosis in any of the several plants tested and that all plants responding with apparent hypersensitivity to $HrpN_{Ech}$ strains also responded to isolated $HrpN_{Ech}$. Several of the plants sensitive to $HrpN_{Ech}$ are also susceptible to bacterial soft rots. This is particularly significant for Saintpaulia, whose interactions with *E. chrysanthemi* have been extensively studied (Barras, F., et al., "Extracellular Enzymes and Pathogenesis of Soft-rot Erwinia," *Annu. Rev. Phytopathol.* 32:201–234 (1994), which is hereby incorporated by reference). Thus, $HrpN_{Ech}$ elicits hypersensitive response-like responses in plants that are susceptible to *E. chrysanthemi* infections under appropriate environmental conditions. The significance of this for the wide host range of the bacterium requires further investigation, and virulence tests with $hrpN_{Ech}$ mutants and additional susceptible plants are needed to determine the general importance of $HrpN_{Ech}$ and the Hrp system in *E. chrysanthemi*. For example, the present data do not address the possibility that other proteins secreted by the Hrp pathway, which are not elicitors of the hypersensitive response in the plants tested, may contribute to pathogenesis in hosts other than witloof chicory.

An important question is whether bacteria expressing heterologous harpins will be altered in pathogenicity. The hrpN genes of *E. chrysanthemi* and *E. amylovora* are particularly attractive for experiments addressing this because of the similarity of the harpins and the dissimilarity of the diseases produced by these bacteria. Unfortunately, attempts to restore the hypersensitive response phenotype to *E. chrysanthemi* and *E. amylovora* hrpN mutants with heterologous hrpN⁺subclones failed. Since the hrpN genes in each subclone successfully complemented hrpN mutations in homologous bacteria and were expressed in heterologous bacteria, the problem is most likely the secretion of the harpins by heterologous Hrp systems. A similar problem has been encountered with heterologous secretion of Pel and cellulase via the Out pathway in *E. chrysanthemi* and *b. carotovora*, species that are more closely related to each other in this rather heterogenous genus than are *E. chrysanthemi* and *E. amylovora* (He, S. Y., et al., "Cloned Erwinia chrysanthemi out Genes Enable *Escherichia coli* to Selectively Secrete a Diverse Family of Heterologous Proteins to its Milieu," *Proc. Natl. Acad. Sci. U.S.A.* 88:1079–1083 (1991); Py, B., et al., "Secretion of Cellulases in *Erwinia chrysanthemi* and *E. carotovora* is Species-specific," *FEMS Microbiol. Lett.* 79:315–322 (1991), which are hereby incorporated by reference).

In conclusion, two classes of proteins contribute to the pathogenicity of *E. chrysanthemi*—a single harpin and a battery of plant cell wall-degrading pectic enzymes. The observation that such a highly pectolytic organism also produces a harpin suggests the fundamental importance of harpins in the pathogenicity of gram-negative bacteria. The observation that a $hrpN_{Ech}$::Tn5-gusA1 mutation reduced the ability of a fully pectolytic strain of *E. chrysanthemi* to initiate lesions in susceptible chicory leaves, but did not reduce the size of lesions that did develop, suggests that $HrpN_{Ech}$ contributes specifically to an early stage of pathogenesis. An attractive possibility is that $HrpN_{Ech}$ releases nutrients to the apoplast for bacterial nutrition before the pel genes are fully expressed (Collmer, A., et al., *"Erwinia chrysanthemi* and *pseudomonas syringae*: Plant Pathogens Trafficking in Virulence Proteins," pages 43–78 in: *Current Topics in Microbiology and Immunology,* Vol. 192: *Bacterial Pathogenesis of Plants and Animals—Molecular and Cellular Mechanisms* (1994), which is incorporated by reference). Patterns of pel and $hrpN_{Ech}$ expression in plants will likely yield further clues to the role of the *E. chrysanthemi* harpin in soft rot pathogenesis.

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 6

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2141 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGATTTTACC CGGGTGAACG TGCTATGACC GACAGCATCA CGGTATTCGA CACCGTTACG      60
GCGTTTATGG CCGCGATGAA CCGGCATCAG GCGGCGCGCT GGTCGCCGCA ATCCGGCGTC     120
GATCTGGTAT TTCAGTTTGG GGACACCGGG CGTCAACTCA TGATGCAGAT TCAGCCGGGG     180
CAGCAATATC CCGGCATGTT GCGCACGCTG CTCGCTCGTC GTTATCAGCA GGCGGCAGAG     240
TGCGATGGCT GCCATCTGTG CCTGAACGGC AGCGATGTAT TGATCCTCTG GTGGCCGCTG     300
CCGTCGGATC CCGCCAGTTA TCCGCAGGTG ATCGAACGTT TGTTTGAACT GGCGGGAATG     360
ACGTTGCCGT CGCTATCCAT AGCACCGACG GCGCGTCCGC AGACAGGGAA CGGACGCGCC     420
CGATCATTAA GATAAAGGCG GCTTTTTTTA TTGCAAAACC GTAACGGTCA GGAACCGTTT     480
CACCGTCGGC GTCACTCAGT AACAAGTATC CATCATGATG CCTACATCGG GATCGGCGTG     540
GGCATCCGTT GCAGATACTT TTGCGAACAC CTGACATGAA TGAGGAAACG AAATTATGCA     600
AATTACGATC AAAGCGCACA TCGCCGGTGA TTTGGCCGTC TCCGGTCTGG GGCTGGGTGC     660
TCAGGGACTG AAAGGACTGA ATTCCGCGGC TTCATCGCTG GGTTCCAGCG TGGATAAACT     720
GAGCAGCACC ATCGATAAGT TGACCTCCGC GCTGACTTCG ATGATGTTTG GCGGCGCGCT     780
GGCGCAGGGG CTGGGCGCCA GCTCGAAGGG GCTGGGGATG AGCAATCAAC TGGGCCAGTC     840
TTTCGGCAAT GGCGCGCAGG GTGCGAGCAA CCTGCTATCC GTACCGAAAT CCGGCGGCGA     900
TGCGTTGTCA AAAATGTTTG ATAAAGCGCT GGACGATCTG CTGGGTCATG ACACCGTGAC     960
CAAGCTGACT AACCAGAGCA ACCAACTGGC TAATTCAATG CTGAACGCCA GCCAGATGAC    1020
CCAGCGTAAT ATGAATGCGT TCGCCAGCGG TGTCAACAAC GCACTGTCGT CCATTCTCGG    1080
CAACGGTCTC GGCCAGTCGA TGAGTGGCTT CTCTCAGCCT TCTCTGGGGG CAGGCGGCTT    1140
GCAGGGCCTG AGCGGCGCGG GTGCATTCAA CCAGTTGGGT AATGCCATCG GCATGGGCGT    1200
GGGGCAGAAT GCTGCGCTGA GTGCGTTGAG TAACGTCAGC ACCCACGTAG ACGGTAACAA    1260
CCGCCACTTT GTAGATAAAG AAGATCGCGG CATGGCGAAA GAGATCGGCC AGTTTATGGA    1320
TCAGTATCCG GAAATATTCG GTAAACCGGA ATACCAGAAA GATGGCTGGA GTTCGCCAAA    1380
GACGGACGAC AAATCCTGGG CTAAAGCGCT GAGTAAACCG GATGATGACG GTATGACCGG    1440
CGCCAGCATG GACAAATTCC GTCAGGCGAT GGGTATGATC AAAAGCGCGG TGGCGGGTGA    1500
TACCGGCAAT ACCAACCTGA ACCTGCGTGG CGCGGGCGGT GCATCGCTGG GTATCGATGC    1560
GGCTGTCGTC GGCGATAAAA TAGCCAACAT GTCGCTGGGT AAGCTGGCCA ACGCCTGATA    1620
ATCTCTGCTC GCCTGATAAA GCGCAAACGA AAAAAGAGAC GGGGAAGCCT GTCTCTTTTC    1680
TTATTATGCG GTTTATGCGG TTACCTGGAC CGGTTAATCA TCGTCATCGA TCTGGTACAA    1740
ACGCACATTT TCCCGTTCAT TCGCGTCGTT ACGCGCCACA ATCGCGATGG CATCTTCCTC    1800
GTCGCTCAGA TTGCGCGGCT GATGGGGAAC GCCGGGTGGA ATATAGAGAA ACTCGCCGGC    1860
CAGATGGAGA CACGTCTGCG ATAAATCTGT GCCGTAACGT GTTTCTATCC GCCCCTTTAG    1920
CAGATAGATT GCGGTTTCGT AATCAATATG GTAATGCGGT TCGGCCTGTG CGCGGGCCGG    1980
GATCACCACA ATATTCATAG AAAGCTGTCT TGCACCTACC GTATCGCGGG AGATACCGAC    2040
AAAATAGGGC AGTTTTTGCG TGGTATCCGT GGGCTGTTCC GGCCTGACAA TCTTGAGTTC    2100
GTTCGTCATC ATCTTTCTCC ATCTGGGCGA CCTGATCGGT T                        2141
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(2) INFORMATION FOR SEQ ID NO:2:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 338 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gln Ile Thr Ile Lys Ala His Ile Gly Gly Asp Leu Gly Val Ser
1 5 10 15

Gly Leu Gly Ala Gln Gly Leu Lys Gly Leu Asn Ser Ala Ala Ser Ser
20 25 30

Leu Gly Ser Ser Val Asp Lys Leu Ser Ser Thr Ile Asp Lys Leu Thr
35 40 45

Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu
50 55 60

Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser
65 70 75 80

Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys
85 90 95

Ser Gly Gly Asp Ala Leu Ser Lys Met Phe Asp Lys Ala Leu Asp Asp
100 105 110

Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln
115 120 125

Leu Ala Asn Ser Met Leu Asn Ala Ser Gln Met Thr Gln Gly Asn Met
130 135 140

Asn Ala Phe Gly Ser Gly Val Asn Asn Ala Leu Ser Ser Ile Leu Gly
145 150 155 160

Asn Gly Leu Gly Gln Ser Met Ser Gly Phe Ser Gln Pro Ser Leu Gly
165 170 175

Ala Gly Gly Leu Gln Gly Leu Ser Gly Ala Gly Ala Phe Asn Gln Leu
180 185 190

Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala
195 200 205

Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val
210 215 220

Asp Lys Gln Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp
225 230 235 240

Gln Tyr Pro Gln Ile Phe Gly Lys Pro Gln Tyr Gln Lys Asp Gly Trp
245 250 255

Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys
260 265 270

Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln
275 280 285

Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr
290 295 300

Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala
305 310 315 320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala
325 330 335

Asn Ala

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGACCTGCAG CCAAGCTTTC C 21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGTACCATGG TTATTACGAT CAAAGCGCAC 30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGATCTCGAG GGCGTTGCCC AGCTTACC 28

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1023 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGCAAATTA CGATCAAAGC GCACATCGGC GGTGATTTGG GCGTCTCCGG TCTGGGGCTG 60

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AAACTGAGCA GCACCATCGA TAAGTTGACC TCCGCGCTGA CTTCGATGAT GTTTGGCGGC 180

GCGCTGCCGC AGGGCTGGG CGCCAGCTCG AAGGGCTGG GGATCAGCAA TCAACTGCGC 240

CAGTCTTTCG GCAATGGCGC GCAGGGTGCG AGCAACCTGC TATCCGTACC GAAATCCGGC 300

GGCGATGCGT TGTCAAAAAT GTTTGATAAA GCGCTGGACG ATCTGCTGGG TCATGACACC 360

GTGACCAAGC TGACTAACCA GAGCAACCAA CTGGCTAATT CAATGCTGAA CGCCAGCCAG 420

ATGACCCAGG GTAATATGAA TGCGTTCGGC AGCGGTGTGA ACAACGCACT GTCGTCCATT 480

CTCGGCAACG GTCTCGGCCA GTCGATGAGT GGCTTCTCTC AGCCTTCTCT GGGGGCAGGC 540

GGCTTGCAGG GCCTGAGCGG CGCGGGTGCA TTCAACCAGT TGGGTAATGC CATCGGCATG 600

GCCGTGGGGC AGAATGCTGC GCTGAGTGCG TTGAGTAACG TCAGCACCCA CGTAGACGGT 660

AACAACCGCC ACTTTGTAGA TAAAGAAGAT CGCGGCATGG CGAAAGACAT CGGCCAGTTT 720

ATGGATCAGT ATCCGGAAAT ATTCGGTAAA CCGCAATACC AGAAAGATGG CTGGAGTTCG 780

-continued

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CCGAAGACGG ACGACAAATC CTGCGCTAAA GCGCTGAGTA AACCGGATGA TGACGGTATG     840
ACCGCGCCCA GCATGGACAA ATTCCGTCAG GCGATCGGTA TGATCAAAAG CGCGGTGGCG     900
GGTGATACCG GCAATACCAA CCTGAACCTG CGTGGCGCGG GCGGTGCATC GCTGGGTATC     960
GATGCGGCTG TCGTCGGCGA TAAAATAGCC AACATGTCGC TGGGTAAGCT GGCCAACGCC    1020
TGA                                                                  1023
```

What is claimed:

1. An isolated DNA molecule encoding a protein or polypeptide corresponding to a protein or polypeptide in *Erwinia chrysanthemi* which elicits a hypersensitive response in plants, wherein said isolated DNA molecule has the nucleotide sequence of SEQ. ID. No. 6.

2. An isolated DNA molecule according to claim **1**, wherein the protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 2.

3. An isolated DNA molecule according to claim **1**, wherein the protein or polypeptide has a molecular weight of 32 to 36 kDa.

4. An isolated DNA molecule according to claim **1**, wherein the protein or polypeptide is heat stable, has a glycine content of greater than 16%, and contains no cysteine.

5. An expression system comprising the isolated DNA molecule according to claim **1** in a vector heterologous to the DNA molecule.

6. An expression system according to claim **5**, wherein the DNA molecule is inserted into the vector in proper sense orientation and correct reading frame.

7. A host cell transformed with a heterologous DNA molecule according to claim **1**.

8. A host cell according to claim **7**, wherein the DNA molecule is inserted into a heterologous expression system.

9. A transgenic plant containing the DNA molecule according to claim **1**.

10. A transgenic plant according to claim **9**, wherein the plant is selected from the group consisting of dicots and monocots.

11. A transgenic plant according to claim **10**, wherein the plant is selected from the group consisting of rice, wheat, barley, rye, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum, and sugarcane.

12. A transgenic plant according to claim **10**, wherein the plant is selected from the group consisting of *Arabidopsis thaliana, saintpaulia, petunia, pelargonium,* and *zinnia.*

13. A method of imparting pathogen resistance to plants comprising:

transforming a plant with the DNA molecule of claim **1** with a pathogen inducible promoter in a plant transformation vector.

14. A method according to claim **13**, wherein the protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 2.

15. A method according to claim **13**, wherein the plant is selected from the group consisting of dicots and monocots.

16. A method according to claim **15**, wherein the plant is selected from the group consisting of rice, wheat, barley, rye, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum, and sugarcane.

17. A method according to claim **15**, wherein the plant is selected from the group consisting of *Arabidopsis thaliana, saintpaulia, petunia, pelargonium,* and *zinnia.*

18. A method according to claim **13**, wherein said transforming is Agrobacterium mediated.

19. A method according to claim **13**, wherein said transforming is effected by particle bombardment.

* * * * *

Exhibit 5 - U.S. Patent No. 6,174,717 to Beer et al.

US006174717B1

(12) **United States Patent**
Beer et al.

(10) **Patent No.: US 6,174,717 B1**
(45) **Date of Patent: Jan. 16, 2001**

(54) **ELICITOR OF THE HYPERSENSITIVE RESPONSE IN PLANTS**

(75) Inventors: **Steven V. Beer**; **Zhong-Min Wei**; **David W. Bauer**; **Alan Collmer**; **Sheng-Yang He**; **Ron Laby**, all of Ithaca, NY (US)

(73) Assignee: **Cornell Research Foundation, Inc.**, Ithaca, NY (US)

(*) Notice: Under 35 U.S.C. 154(b), the term of this patent shall be extended for 0 days.

(21) Appl. No.: **08/851,376**

(22) Filed: **May 5, 1997**

Related U.S. Application Data

(62) Division of application No. 08/200,724, filed on Feb. 23, 1994, now Pat. No. 5,849,868, which is a continuation of application No. 07/907,935, filed on Jul. 1, 1992, now abandoned.

(51) **Int. Cl.**[7] **A01H 1/00**; C07H 21/04; C07K 14/27; C12N 5/14

(52) **U.S. Cl.** **435/252.33**; 435/69.1; 435/71.1; 435/71.2; 435/410; 435/320.1; 435/243; 435/252.33; 435/6; 435/418; 435/419; 435/252.3; 435/252.1; 530/350; 536/23.7; 536/23.1; 536/24.5; 800/278; 800/279; 800/288

(58) **Field of Search** 800/200, 205, 800/278, 279; 435/69.1, 71.1, 71.2, 410, 320.1, 243, 252.33, 6, 418, 419, 252.3, 252.1; 530/350; 536/23.7, 23.1, 24.5

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Primary Examiner—Phuong T. Bui
(74) *Attorney, Agent, or Firm*—Nixon Pebody LLP

(57) **ABSTRACT**

The nucleic acid and amino acid sequences for proteinaceous elicitors of the plant defense reaction known as the hypersensitive response are described along with methods for preparation and processes for inactivation.

22 Claims, 2 Drawing Sheets

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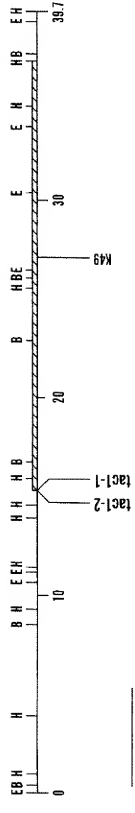

FIG. 1

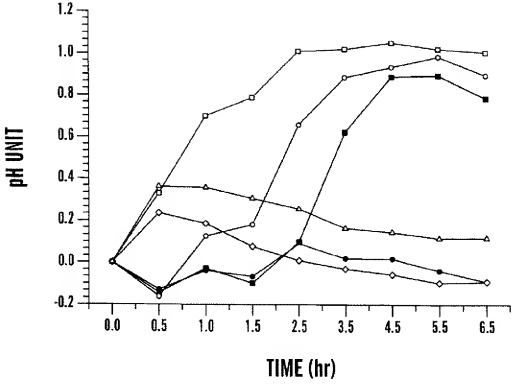

FIG. 2

ELICITOR OF THE HYPERSENSITIVE RESPONSE IN PLANTS

This is a division of application Ser. No. 08/200,724 filed on Feb. 23, 1994, now U.S Pat. No. 5,849,868, which is a continuation of application Ser. No. 07/907,935, filed Jul. 1, 1992, now abandoned.

Partial support for the research which led to the making of the present invention was provided by funds from the United States Department of Agriculture. Accordingly, the United States government has certain statutory rights to this invention under 35 USC 200 et seq.

Plants, as well as humans and animals, suffer injury and losses due to infection by bacteria. On a worldwide basis, bacteria classified in the genera Erwinia, Pseudomonas and Xanthomonas are responsible for most losses due to bacterial plant pathogens. Many of the bacterial diseases of plants cause farmers great losses on a sporadic basis. The losses result from death, disfigurement or reduced productivity of affected plants.

Many bacterial pathogens of plants exhibit a marked degree of specificity towards the plants that they infect. For example, *Erwinia amylovora* infects apples, pears and related plants of the family Rosaceae. Other plants do not become diseased when exposed to *E. amylovora*. However, when sufficient cells of *E. amylovora* are introduced into leaf tissue of the other plants, the mesophyll tissue collapses within hours. This collapse has been called the hypersensitive response (HR), and it is considered a defense reaction of plants since, during the HR, the bacteria are delimited within the collapsed tissue, eventually die, and thus do not cause much damage to the plant as a whole.

The genes that bacterial plant pathogens require for HR-eliciting ability, are called hrp genes, for hypersensitive reaction and pathogenicity, are also required for causing disease. However, the products of hrp genes and how they function in elicitation of the HR, and in disease development, remained unknown prior to the present invention. The present invention concerns products of hrp genes (elicitors) that are responsible for the collapse seen in the HR and are required for disease development.

Interactions between bacterial pathogens and their plant hosts generally fall into two categories: (1) compatible (pathogen-host), leading to intercellular bacterial growth, symptom development and disease development in the host plant; and (2) incompatible (pathogen-nonhost), resulting in the hypersensitive response, a particular type of incompatible interaction occurring, without progressive disease symptoms. During compatible interactions on host plants, bacterial populations increase dramatically and progressive symptoms occur; during incompatible interactions bacterial populations do not increase, and progressive symptoms do not occur.

The hypersensitive response of higher plants is characterized by the rapid, localized collapse and death of tissues containing an incompatible pathogen (a microorganism that is pathogenic only on other plants) and is associated with the defense of plants against many bacteria, fungi, nematodes, and viruses [see Phytopathogenic Prokaryotes, (M. S. Mount and G. H. Lacy eds.) Academic Press, New York. pp 60 149–177 (1982)]. Elicitation of the hypersensitive response by bacteria was first demonstrated in 1963 when the intercellular spaces of tobacco leaves were infiltrated with 10^7 cells/ml of an incompatible pathogen. The infiltrated areas collapsed within 24–48 hours and ceased to support bacterial multiplication [see Nature 199:299 (1963)]. Thus, in the HR, the pathogen is localized and further growth is restricted.

The technique used in the laboratory to demonstrate the HR is straight-forward. The intercellular spaces of tobacco leaves are infiltrated by first puncturing a sector on a leaf with a common straight dissecting needle. Then a 1 ml capacity syringe (without a needle), containing 0.1–0.5 ml of a bacterial cell suspension (usually 10^7–10^8 viable cells/ml) of bacteria is pressed against one side of the leaf directly over the puncture. While pressing a finger on the opposite side of the leaf to stabilize it and to prevent liquid from leaking out of the punctured area, the syringe plunger is pressed gently to introduce the bacterial suspension into the leaf. Infiltration is considered successful when a water-soaked area approximately 1–4 cm^2 appears in the leaf.

A common hypothesis proposed to explain the mechanism of hypersensitive reaction induction involves the production by bacteria of a specific elicitor that reacts with a specific receptor on the plant cell. However, the molecular basis (gene and gene product) for this response to potential pathogens had been unknown prior to the present invention in spite of continued research by plant pathologists since the HR first was described in 1963.

Physiological and genetic observations suggest that the same bacterial factor that elicits the hypersensitive response in nonhosts is also required for pathogenicity in hosts.

Production of the elicitor of the hypersensitive response is controlled by a cluster of several hrp genes, which are highly conserved, and often interchangeable, among many species of plant pathogenic bacteria. Although individual and several hrp genes have been cloned by others, functional clusters of hrp genes have been cloned only from *Erwina amylovora* and *Pseudomonas syringae*. These clusters have been shown to confer on nonpathogenic bacteria the ability to elicit the hypersensitive response in tobacco and other leaves [see Mol. Plant-Microbe Interact. 4:132 (1991), J. Bacteriol 170:4748 (1988); and Beer et al., Advances in Molecular Genetics of Plant-Microbe Interactions (H. Hennecke and D. P. S. Verma eds.) Kluwer Academic Publishers, Boston, pp 53–60 (1991)].

The elicitor, according to the present invention, was initially isolated and purified from *E. coli* DH5α(pCPP430), and later from a wild-type strain of *E. amylovora*, the bacterium that causes a disease of rosaceous plants, such as apple and pear, known as fire blight. According to the present invention, the name "harpin" is proposed for the hypersensitive response elicitor from *E. amylovora*; this elicitor is considered to be the archetype for a family of proteinaceous HR elicitors that are produced by many different phytopathogenic bacteria.

It is thus one aspect of this present invention to describe specific elicitor proteins isolated from bacteria, which when applied to nonhost plants, cause a toxic response that is similar to the response elicited by living cells of the bacteria that produced the proteins. A further aspect of this present invention is to isolate and describe the genes that encode the elicitor proteins, which might be used to cause plants or other organisms to produce elicitor protein, which would exert its toxic effects in a precise controlled manner.

A further aspect of this present invention is to provide sufficient characterization, and identification of these proteins to allow design and development of techniques that will inactivate, destroy, or bind with these proteins. This aspect is desirable because it is known the same proteins are required by the bacteria that produce them in order to cause disease in host plants of the bacteria. Neutralizing the toxic effects of the proteins neutralizes their roles in disease and reduces disease in plants.

A still further aspect of the present invention is to develop antibodies against these proteins, sequence the

antibodies produced, construct nucleic acid sequences which when inserted properly into the genome of a plant would cause the plant to express the antibody and thus prevent bacteria from causing disease in plants.

One portion of the present invention is based on the identification of a particular hrp gene of the hrp gene cluster of *Erwinia amylovora*. That particular gene is transcribed and translated to yield the proteinaceous elicitor of the of the hypersensitive response. Another portion of the present invention deals with the identification of homologous genes from Erwinia, Xanthomonas, and Pseudomonas species that encode similar proteins to the HR elicitor from *E. amylovora*. Prior to the making of the present invention, the isolation of a proteinaceous elicitor of the hypersensitive response had not been reported. Thus, another portion of the present invention is a description of techniques for isolation and purification of a proteinaceous elicitor of the hypersensitive response. An additional portion of this invention concerns the genetic manipulation of the genes encoding the HR-elicitor proteins to enhance production of harpin.

Therefore, it may be summarized that the various portions and aspects of the present invention relate to providing prophylaxis against *Erwinia amylovora*, the causative agent of fire blight of apple, pear, and other rosaceous plants. In addition, the present invention broadly relates to providing prophylaxis to bacteria of the genera Erwinia, Pseudomonas and Xanthomonas which cause other diseases of a variety of plants.

In order to provide a clear understanding of the present invention, the following terms relating to the bacterial strains, cosmids and plasmids referred to in the description of the present invention are provided.

DH5α	A laboratory strain of *Escherichia coli* used routinely for cloning:
Ea321	Wild-type strain of *Erwinia amylovora* from which all mutants and clones were derived;
Ea321T143	Hrp mutant containing transposon Tn5 that was used to screen a cosmid liberry (in pCPP9 vector) for restoration of Hrp function. This screening resulted in the identification of cosmid pCPP430. (This mutant has an insertion in one of the hrp genes, not hrpN; the effect of the insertion is to prevent expression of harpin by mutagenesis of the operon).
Ea321K49	Hrp mutant containing the Tn10mini-kan transposon which is inserted in an hrp gene involved in regulation of harpin production.
Ea321T5	Hrp mutant containing the hrpN gene that was mutagenized with the TnSinc1 nonpolar transposon. (This mutant of Ea321 has no insertion in the gene that encodes harpin).
pCPP9	A cosmid vector constructed for the cloning of DNA of *E. amylovora*. The vector portion of pCPP430.
pCPP430	Cosmid containing 46.5 kb of Ea321 DNA that includes the whole hrp gene cluster of Ea321. This cosmid bestows on *E. coli* the ability to elicit the HR, and restore the Hrp phenotype to all Hrp mutants of *E. amylovora*. Clone from which hrpN was derived.
pCPP1084	Plasmid containing a 1.3 kb HindIII fragment from pCPP430, which includes the whole hrpN (1155 base pairs). The vector is pBluescript M13+.
pCPP50	A plasmid developed by modifying pNIH[GS]-A2 of Masui et al. (Bio/Technology, January 1984 pp. 81–85). A fragment of the original was deleted and a fragment from pBluescript was inserted. The modifications were made to create a vector more suitable for harpin production.
pCPP2139	Plasmid that when in *E. coli* results in super-production of harpin. Constructed by cloning the hrpN gene from pCPP430 into pCPP50.

-continued

pBluescript M13+	A plasmid routinely used for subcloning and sequencing of DNA. Used also for in vitro expression of protein from cloned DNA.

In addition, these and other terms used throughout this description may be found in Molecular Plant-Microbe Interactions 4(5):493 (1991) and Advances in Molecular Genetics of Plant-Microbes Interaction 1:53 (1991).

Both *E. coli* DH5α(pCPP1084) and *E. amylovora* Ea321 have been deposited with the American Type Culture Collection in Manassas, Va. Their deposit number are ATCC 69021 and ATCC 49947, respectively. The deposit of ATCC 69021 has been made under the Budapest Treaty, and cultures will be made available in accordance with the provisions of this treaty.

The various aspects regarding the identification, isolation, purification and characterization of the HR elicitor and gene according to the present invention can be more clearly understood from the following figures and examples, all of which are provided for purposes of clarifying the present invention and not for limiting the scope thereof.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a restriction endonuclease map of the hrp cluster of *Erwinia amylovora*; and

FIG. 2 depicts the changes in pH of a bathing solution of tobacco cell-suspension cultures.

More particularly, FIG. 1 represents the restriction endonuclease map of the hrp cluster of *Erwinia amylovora* in which "E" designates EcoRI, "H" designates Hindlul, and "B" designates BamHI restriction sites. The vertical lines indicate the location of transposon insertions that have been tested for their effects on the ability to elicit the HR and to be pathogenic on pear. Metibolically active cells of *Erwinia amylovora* Ea321 [see Molecular Plant-Microbe Interactions 1(3):135 (1988)] and *E. coli* DH5α(pCPP430) I with all indicated insertions fail to elicit the hypersensitive reaction in tobacco. The region encompassed by all indicated insertions is essential also for the elicitation of a K^+/H^+ exchange reaction of tobacco cell suspension cultures. Derivatives of Ea321 containing all the indicated insertions are not pathogenic to pear.

More particularly in respect to FIG. 2, the control values (no additive) were subtracted prior to graphing. Open squares depict harpin (60 nM); open circles depict cells of *E. coli* DH5α(pCPP430) (5×10^7 cells/ml); filled squares depict cells of *E. amylovora* Ea321 (5×10^7 cells/ml; triangles depict cells of *F. coli* DH5α(pCPP430K49) (5×10^7 cells/ml); diamonds depict cells of *E. amylovora* Ea321K49 (5×10^7 cells/ml); and filled circles depict cells of *E. coli* DH5α(pCPP9) (5×10^7 cells/ml). Tobacco cell-suspension cultures were shaken at room temperature with the indicated preparations. The pH was measured at the intervals indicated. All preparations that elicited HR in tobacco leaves also caused a pH increase in the tobacco cell-suspension culture medium.

EXAMPLE I

Plasmid pCPP430 was identified from a library of genomic DNA of the wild-type strain of *E. amylovora*, known in our laboratory as Ea321, and has been deposited in the American Type Culture Collection as 49947. The strain was received in 1978 from the French National

Collection of Phytopathogenic bacteria, in which it is known as CNFB 1367. Genomic DNA was isolated and digested with Sau3A, ligated into the cosmid vector of pCPP9 previously digested with BamHI, packaged and transfected into *E. coli* strain ED8767 according to procedures previously described [see Mol. Plant-Microbe Int. 1:135 (1988)]. The resulting cosmids were mobilized into strains by conjugation with the aid of the helper plasmid pRK2013 [Bauer, D. W., Molecular genetics of pathogenicity of *Erwinia amylovora*: techniques, tools and their application. Ph.D. thesis. Cornell University, Ithaca, N.Y. (1989)].

The resulting library was diluted and spread on plates of nutrient agar containing both spectinomycin and kanamycin 50 μg/ml final concentration. Plates containing about 500 colonies, after incubation at 37° for 24 hr, were selected when the diameter of each colony was 0.5–1.0 mm. The colonies from these plates were replica-stamped onto plates containing Luria-Bertani agar (LA) on which 0.1 ml of a suspension of strain Ea321T143 previously had been spread. Ea321T143 is a Tn10-induced Hrp⁻ mutant strain of Ea321; it is not pathogenic to pear fruit and does not elicit the HR in tobacco and other plants. It had been grown to $O.D._{620}$=1.3 in Luria broth plus tetracycline (10 μgm/ml). The LA plates were incubated for 5 hr at 28° C. and the growth on these plates were replica-plated on to a minimal medium for the growth of *Erwinia amylovora*, which contained glucose 2 g/l, asparagine 1.5 g/l. sodium citrate 0.25 g/l, $MgSO_4$ 5 mg/l, nicotinic acid 0.25 g/l, $(NH_4)_2SO_4$ 1 g/l, K_2HPO_4 3.51 g/l, KH_2PO_4 1.51 g/l, and 50 mg/l spectinomycin and 10 mg/l tetracycline. This procedure selected transconjugants of Ea321T143 which contained various cosmids of the Ea321 library. After 48 hr of incubation at 28° C., freshly cut slices of immature pear fruit were pressed onto the surface of each plate of transconjugants such that all colonies beneath the pear-slice came in contact with pear tissue. The pear slices were inverted, incubated in plastic boxes lined with well-moistened paper towels and observed daily for up to 5 days for the presence of droplets of ooze. The immature pear fruit had been harvested approximately 6 weeks following bloom, from trees of *Pyrus communes* cv. Bartlett. The fruits were 2–4 cm in diameter, and they were stored at 0–2° C. until used. Ooze as used in this description of the present invention, is a mixture of plant and bacterial products that consists largely of living bacterial cells.

The ooze was dilution-streaked on plates of *E. amylovora*-minimal medium with 50 μg/ml spectinomycin and 10 μgm/ml tetracycline, incubated for 2 days at 28° C. and individual colonies were picked with sterile toothpicks, propagated on a fresh plate of Ea minimal agar+50 μg/ml spectinomycin and 10 μgm/ml tetracycline and retested for pathogenicity. Freshly cut pear fruit tissue was stabbed with toothpicks contaminated with the strains to be tested. Cosmids from those colonies which caused disease on pear fruit were remobilized into DH5α from Ea321T143 by combining 0.5 aliquots of overnight LB+antibiotic cultures of DH5α, the Ea321T143 path⁻ transconjugant (the strain of Ea321T143 containing the cosmid which bestowed on Ea321T143 the ability to cause disease), and pRK2013, the helper plasmid. The combination was mixed thoroughly, centrifuged, and the pellet suspended in 150 μl of L broth, without antibiotics. The pellet was thoroughly resuspended and 0.1 ml drops were placed on LA plates, allowed to soak into the agar without spreading, and then the plates were incubated at 28° C. for 5 hr. After incubation, the spotted growth was resuspended in 1 ml of 5 mM potassium phosphate buffer, pH 6.5, and 0.1 aliquots were spread onto plates of LA+50 μg/ml spectinomycin and 20 μg/ml Nalidixic acid, which were then incubated for 48 hr at 37° C. Colonies were simultaneously transferred with toothpicks to plates of LA+50 μg/ml spectinomycin and LA+Km. Those colonies that grew ooily on the 50 μg/ml spectinomycin plates, indicating loss of the helper plasmid pRK2013 (Km^s) were chosen for preservation by freezing and for further study.

To determine if the same cosmid that restored pathogenicity to pear, hereinafter referred to as pCPP430, also affected the reaction of Ea321T143 on tobacco, suspensions were infiltrated into tobacco leaf sectors. The effect of pCPP430, maintained in *E. coli* DH5α was tested in tobacco. The strain was grown to OD_{620} 0.4–0.6 (approximately 10^8 cfu/ml) in Luria broth+50 μg/ml spectinomycin. The culture was centrifuged (12,000xg for 1 minute), resuspended in 5 mM phosphate buffer pH 6.5, to the original volume and infiltrated into tobacco leaves. Collapse of the tissue occurred within 8 hrs. No collapse was observed when cells of DH5α(alone) or DH5α(pCPP9) were infiltrated into tobacco leaves. Thus, we concluded that pCPP430, containing particular DNA of Ea321 enabled *E. coli* DH5α to cause the HR reaction and that pCPP430 contained all the genes necessary for this reaction.

The hrp gene from *E. amylovora* contained in the cosmid pCPP430, is particularly well expressed in *Escherichia coli* [see Advances in Molecular Genetics of Plant-Microbe Interactions, supra; Phytopathology 79:1166 (1989); and Mol. Plant-Microbe Interactions 4(5):493 (1991)]. Usually de novo RNA and protein synthesis was required for Ea321 to elicit the HR. However *E. coli*(pCPP430) and Ea321 (pCPP430) are able to elicit the HR in the presence of bacterial transcriptional or translational inhibitors such as rifampicin and tetracycline. This indicated that the HR elicitor was present in/on cells of *E. coli* DH5α(pCPP430) before tobacco leaves were infiltrated with the bacteria.

The search for the HR elicitor began by infiltrating tobacco leaves with the cell-free culture supernatants of *E. amylovora* Ea321, Ea321(pCPP430) or *E. coli* DH5α (pCPP430). The supernatants were produced by growing each strain in LB broth with the appropriate antibiotic to late log phase ($O.D._{620}$=ca. 1.0). As we expected, based upon the experience of other workers [see Phytopathology 57:322 (1967)], no hypersensitive response occurred.

Strain Ea321(pCPP430) was created by the following procedure:

EXAMPLE II

Strains Ea321, *E. coli* DH5α(pCPP430) and *E. coli* DH5α (pRK2013) were grown overnight in LB broth containing respectively, no antibiotic, 50 μg/ml spectinomycin, or Km^{50}. The next morning 0.5 ml aliquots of each strain were combined in a microcentrifuge tube, centrifuged for 2 min and resuspended in 0.15 ml of Luria broth (no antibiotics). A 0.1 ml aliquot of this suspension was spotted on Luria agar (no antibiotics) and incubated for 5 hr at 28° C. The growth from this spot was resuspended in 1 ml of 5 mM potassium phosphate buffer, pH 6.5, and 0.1 ml aliquots were spread on plates of *E. amylovora* minimal medium containing 50 μg/ml spectinomycin to select for strains of Ea321 harboring pCPP430. Plates were incubated for 2–3 days at 28° C. Individual colonies were toothpicked simultaneously to minimal medium containing 50 μg/ml spectinomycin and to minimal medium containing Km^{50}. Only those colonies that grew on the medium with 50 μg/ml spectinomycin (indicating selection of pCPP430) but not on the medium with Km^{50} (indicating loss of the helper plasmid pRK2013) were selected for further study.

Although cell-free culture supernatants of all bacteria used failed to elicit the hypersensitive response, preparations of certain cells in a new manner resulted in cell-free preparations that elicited a strong hypersensitive response within 12 hours that was indistinguishable from that elicited by whole metabolizing bacterial cells from which the preparations were made. The elicitor of the hypersensitive response was isolated, purified and characterized from this cell-free elicitor preparation (CFEP) according to Example III.

The isolation of CFEP containing harpin from *E. coli* DH5α(pcPP430) according to the present invention is described in the following example:

EXAMPLE III

Cells of *E. coli* DH5α(pCPP430) were grown in Luria-Bertani (LB) medium to OD_{620}=0.8, collected by centrifugation and resuspended in one tenth the original volume of 5 mM potassium phosphate buffer, pH 6.5, with 0.1 mM phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor. The cells were then disrupted by sonication using a Sonicator Ultrasonic Cell Disruptor™ (Heat System-Ultrasonics) at a power output of 4, and the pulsar cycle timer set to 40% duty cycle (under these conditions, 10 ml of bacterial suspension were sonicated for 10 min on ice). After the debris from sonication were removed by centrifuging at 12,000xg for 1 hour, the supernatant liquid was filtered through a 0.2 μm pore-size membrane filter to remove any remaining intact cells. The resulting preparation, at dilutions up to about 1:10, was able to elicit the hypersensitive response in tobacco leaves. The CFEP contained the intracellular material from a culture of OD_{620}= 0.4, the same density of living cells of *E. coli* required for elicitation of the hypersensitive response.

The purification of harpin according to the present example is described in the following example:

EXAMPLE IV

Initial experiments using the preparation obtained from Example III indicated that the HR-eliciting activity was heat stable and proteinaceous in nature. The preparation retained HR-eliciting activity as determined by infiltration of tobacco leaves as described previously following incubation overnight at 65° C. However, unless PMSF, the serine protease inhibitor, had been added during preparation, all HR-eliciting activity was lost after 3 hours at 37° C. or 6–8 hours at 4° C. Incubation of the preparation with Pronase E (Sigma) at 100 μg/ml, for 1 hour at 37° C. destroyed any elicitor activity.

The advantage of the heat stability of the elicitor preparation was used to aid in further purification of the elicitor. Only a limited number of proteins remained after holding the elicitor preparation of Example III in a boiling water bath for 10 minutes and subsequent removal of the insoluble material by centrifugation. One band, corresponding to 44 kD, was prominent following electrophoresis of the heated Example III preparation on SDS-polyacrylamide (10% SDS-PAGE gels were prepared and used according to instructions of the supplier, Hoeffer Scientific Instruments; protein in the gels was stained with 0.025% Coomassie Blue R-250 for 30 min and destained with 50% methanol and 10% acetic acid solution) gels. A band of this mobility was uniquely present in all preparations with HR-eliciting activity. Following resolution of the Example III preparation on an isoelectric-focusing granulated gel bed or by ion-exchange chromatography the fractions with HR-eliciting activity always contained a protein that corresponded to 44 kD in molecular size with a pI of 4.0 to 4.5.

To accomplish further purification of harpin, several separation techniques were applied to CFEPs prepared as discussed in Example III. Before each step CFEP was heated in a boiling water bath for 10 minutes, cooled to 25–30° C. and centrifuged for 10 min at 12,000xg. The supernatant liquid was retained and filtered through a 0.2 μm pore size filtration membrane (Millipore, MF).

The heat-treated CFEP was bound to an anion exchange resin (Whatman DE-52) and eluted stepwise with increasing amounts of KCl in 5 mM potassium phosphate buffer, pH 6.5. Harpin was eluted from the column by buffer containing 90 mM KCl. The presence of harpin was determined by infiltration of tobacco leaf sectors with elements from the column that had been concentrated to 50% of the initial volume. In addition, fractions were electrophoresed in SDS-PAGE gels according to standard procedures. Final purification was accomplished by High Pressure Liquid Chromatography (HPLC). Preparations purified by ion-exchange chromatography were adjusted to pH 2 by the addition of acetic acid and, following centrifugation to remove any precipitates, were applied to a reverse-phase HPLC prepacked column (YMC AQ-303). The column was eluted with a gradient of 10–70% acetonitrile at pH 2 in 0.25% w/v trifluoroacetic acid. Detection of protein was by absorption of light from 190 nm to 300 nm. Each 0.25 ml fraction was tested for ability to elicit the HR by infiltration of tobacco leaf sectors.

The granulated gel bed used for the resolution of the Example III preparation was prepared with Bio-lyte™ (Bio-Rad Laboratories) as recommended by the manufacturer. Wide-range ampholytes, pH 3–10 (Sigma) were used at a final concentration in the slurry of 2%. Electrode solutions were 1M H_3PO_4 (anode) and 1M NaOH (cathode).

To determine whether the prominent 44 kD protein ("harpin") band present in all HR-eliciting samples, had elicitor activity, the appropriate unstained region of a preparative SDS-gel was cut and electroeluted with buffer lacking SDS. The eluted protein (200 μg/ml) was dialyzed overnight against 2 liters of 5 mM potassium phosphate buffer, pH 6.5, containing 0.1 mM phenylmethyl sulfonyl fluoride. At concentrations ≧500 nM (≧25 μg/ml), harpin elicited the hypersensitive response in leaves of all plants tested, including tobacco, tomato, and *Arabidopsis thaliana*.

Subsequent experimentation confirmed that harpin was protease sensitive, heat-stable, and acidic. Treatment of harpin with protease abolished HR-eliciting ability and eliminated the 44 kD protein band from SDS polyacrylamide gels. However, when harpin was incubated with protease that had been held at 100° C. for 10 min to inactivate the enzyme, the preparation retained HR-eliciting activity. When active protease was present in the infiltration mixture, no hypersensitive response developed. However, infiltration of tobacco leaves with active or heat-inactivated protease alone did not result in any macroscopic symptoms. Harpin retained its HR-eliciting activity following heating in a boiling water bath for 10 min. Purified harpin from an SDS gel had a pI of 4.3 as determined by resolution ort thin-layer isoelectrofocusing gels using conventional techniques.

The subcellular location of harpin according to the present invention is described in the following example:

EXAMPLE V

The location of harpin on the organism's cell surface was suggested by the following observations: (i) the supernatant of *E. amylovora* Ea321(pCPP430) or *E. coli* DH5α (pCPP430) did not elicit the hypersensitive response, indi-

cating that harpin is not secreted into the medium but rather is present in or on the bacteria ; (ii) following incubation at 37° C. for 5 min of whole cells of Ea321(pCPP430) and *E. coli* DH5α(pCPP430) with 40 and 80 μg/ml of protease, respectively, and with 40 μg/ml tetracycline to halt the continued production of harpin, the bacteria failed to elicit a hypersensitive response. When 0.5 mM of PMSF, the protease inhibitor, was included in the above incubation mixture, the bacteria elicited the hypersensitive response; PMSF apparently protected harpin from inactivation by protease. (Infiltration of tobacco leaves with PMSF or tetracycline alone had no effect, indicating that neither compound functions independently in causing HR); (iii) treatment of bacteria with increasing amounts of protease resulted in decreased ability to elicit the hypersensitive response which correlates well with the disappearance of harpin from SDS gels in which preparations from the protease-treated bacteria had been electrophoresed [Table 1]: (iv) following centrifugation of the Example III preparation at 105,000×g for 1 hr, most HR-eliciting activity was found in the supernatant liquid, however, when 30 mM $MgCl_2$, a membrane stabilizer, was added before sonication, most activity was associated with the pellet, that is with the centrifuged portion containing the membranes; and (v) gel-permeation chromatography of unboiled Example III preparation indicated association of the elicitor with a very high molecular weight (>10^6 D) fraction which were probably membrane vesicles; and (vi) fractionation of lysed cells of Ea321(pCPP430) [see Science 233:1403 (1985)] in the ultracentrifuge and reaction with a harpin-specific antibody, resulted only in reaction with the cell membrane faction and the whole cell control.

The foregoing results indicate that harpin is located at or near the bacterial cell-surface, and that it is unstable. Cell suspensions of Ea321(pCPP430) or *E. coli* DH5α (pCPP430) maintain their HR-eliciting activity for not more than 0.5 hr and 1 hr, respectively, in the presence of tetracycline (40 μg/ml), a translation inhibitor. In addition, harpin was not detected once the cells lost HR-eliciting activity. However, when the protease inhibitor PMSF (0.5 mM) was included in the suspension, the bacteria retained HR-eliciting activity for more than two hours, and decreasing amounts of harpin were detected simultaneously in the SDS gels over time. On an equal cell number basis, more protease was required to destroy harpin and prevent the hypersensitive reaction for *E. coli* DH5α(pCPP430) than for Ea321(pCPP430). Thus, the sensitivity of harpin to proteolysis may explain the previous observations of the short-lived nature of the HR-eliciting ability of phytopathogenic bacteria [see Science 245:1374 (1989)].

The following procedure and Table 1 depict the protocol for, and results of, protease sensitivity of HR-eliciting activity from *E. amylovora* Ea321 containing its hrp gene cluster.

Cells of *E. amylovora* Ea321(pCPP430) were grown in LB medium and harvested at $O.D._{620}$=0.6 by centrifugation. The cells were then resuspended in 0.1 volume of 5 mM potassium phosphate buffer, pH 6.5, containing 40 μg/ml tetracycline. Protease (as indicated in Table 1) was added to 200 μl cell suspension and incubated at 37° C. for 5 minutes and 100 μl of each mixture was subsequently infiltrated into tobacco leaves. Collapse was noted 24 hrs after infiltration. 20 μl of 5× cracking buffer was mixed with 80 μl of the remaining mixtures, boiled for 5 minutes and then centrifuged for 10 min in a microcentrifuge, prior to loading 15 μl in each lane of a 10% SDS-PAGE gel. Electrophoresis was carried out for 2 hours at 20 mA, followed by staining with 0.025% Coomassie Blue R-250 for 30 min and destaining with 50% methanol and 10% acetic acid solution. Cell-free supernatant, produced from the LB culture, was filter-sterilized and then concentrated to one tenth the original volume with the Centriprep-10 (Amicon). Treatment with the higher levels of protease resulted in loss of HR-eliciting ability and disappearance of the harpin band (44 kD) from the SDS gels. The resulting data from this protocol are reported in the following table:

TABLE 1

Protease/ml	HR-elicitation on Tobacco	Harpin Detected
0 μg	+	+
5 μg	+	+
10 μg	+	+
20 μg	weak	+
40 μg	–	–
80 μg	–	–
80 μg + 0.5 mM PMSF	+	+
cell-free supernatant	–	–

+ = a positive reaction;
– = a negative reaction.

The ability of bacterial strains to elicit the hypersensitive response in intact tobacco leaves is strongly correlated with their ability to elicit a K^+/H^+ exchange reaction in tobacco cell suspension cultures. The two reactions are related genetically, as a major portion of hrp gene cluster of *E. amylovora* is needed for elicitation of the K+/H+ exchange reaction. Thus, the effect of harpin on tobacco cell suspension cultures was tested according to the following example.

The effect of harpin on plants, plant cells and tissues according to the present invention is described in the following example:

EXAMPLE VI

To determine if a particular preparation had HR-eliciting activity, we used a technique similar to that used with whole bacterial cells [see Mol. Plant-Microbe Interact. 4:494 (1991)]. Tobacco plants (*Nicotiana tabacum* L. 'Xanthi') were grown in artificial soil mix to a height of 90–100 cm. Plants were moved from the greenhouse to the laboratory <24 hr before infiltration. Infiltration of the leaf lamina was done with a needle-less syringe through a small hole made with a dissecting needle. Collapse of the infiltrated area, indicative of the HR, was recorded 24 hrs after infiltration.

All CFEPs that contained the 44 kD protein, as detected by SDS-PAGE, caused collapses of the infiltrated areas of the tobacco leaves. Harpin, purified by HPLC (Example IV) elicited the HR at concentrations ≧500 nM.

To test the effect of harpin on tobacco cell suspension cultures, four-day old tobacco cell suspension cultures (*Nicotiana tabaccum* var. Samsun) were obtained from the Biotechnology Program at Cornell University. The cell suspension was filtered through a single layer of loose weave cheesecloth into a 1 liter beaker to eliminate any large clumped masses. Tobacco Assay Medium [MES 0.5 mM, mannitol 0.175 M, K_2SO_4 (2 ml of a 0.25 M stock solution), $CaCl_2$ (2 ml of a 0.25 M stock solution) high-purity water 996 ml; adjusted to pH 6.0 with 1N NaOH and filtered through a 0.2 μm pore-size membrane filter] was used to wash as many cells as possible through a single layer of cheesecloth. This washed and strained suspension was next poured into a large funnel lined with 1 layer of Miracloth™ (non-woven cloth), and the cells that lined the Miracloth™ were gently washed with an additional 200–400 ml of Tobacco Assay Medium. Fifteen gm of wet cells were

weighed and gently resuspended in 415 ml of Tobacco Assay Medium. Twenty ml aliquots of this suspension were measured in to conical plastic cups (4 cm top diameter; 2.5 cm bottom diameter; 4 cm high) and immediately placed on a rotary shaker set at 150 rpm with a 2 cm stroke and maintained at 25±3° C.

Cells were allowed to equilibrate until they reached a pH of approximately 5.8 (usually 20–30 min). At this point, 1 ml of bacterial suspension, or sonicated extract, or 0.5 ml of purified protein containing 20 μl of a 20 μg/ml concentrate of PMSF was added to each tobacco cell sample. The pH of the sample was read with a Corning pH meter and was adjusted back to pH 6 with 0.1 N NaOH (or 0.1 N HCl as needed). The second reading was taken 30 minutes after the first reading. All subsequent readings were taken at hourly intervals for up to 6 hours after the reading at time 0. All treatments were tested in duplicate.

Bacterial cell suspensions were prepared by growing overnight cultures in LB with the appropriate antibiotic and then diluting the strains back to an OD_{620} of 0.20 the next morning. The cultures were regrown to OD 0.4. At this OD, strains of Ea321 and their derivatives are estimated to have a concentration of approximately 2×10^8 cfu/ml. Strains of *E. coli* DH5α and their derivatives are estimated to have a concentration of approximately 1×10^8 cfu/ml. The cells were centrifuged at 5000xg and resuspended to give 5 fold concentrations (for Ea321 and derivatives) and 10 fold concentrations (for *E. coli* and derivatives) in 1 mM MES buffer pH 6. In this manner, cell concentrations of approximately 1×10^9 cfu/ml were achieved. When 1 ml of cell suspension was added to 20 ml tobacco cell suspension, the final concentration of cfu/ml for the assay was estimated at 5×10^7 per ml.

Cells of *E. amylovora* caused an increase in pH of the bathing solution (a measure of the K^+/H^+ exchange reaction) with a 2–3 hr delay following addition of bacteria to the tobacco cell suspension culture (see FIG. **2**). In contrast, a one-time addition of harpin at time zero caused a rapid increase in the pH of the bathing solution during the first hour. The pH decreased slightly during subsequent incubation. Mutants of *E. amylovora* that do not produce harpin in vitro failed to elicit the K^+/H^+ exchange reaction. Strains of *E. coli* containing mutations in the cloned hrp gene cluster of *E. amylovora* also failed to elicit the exchange reaction. The elicitation of the exchange reaction, as well as the hypersensitive reaction, by harpin provides additional evidence that harpin is active in bacteria-plant interactions. The data from these studies on the effect of harpin on tobacco cell cultures is presented in FIG. **2**.

The following example provides a comparison of harpin obtained from *E. coli* DH5α(pCPP430) and Ea 321.

EXAMPLE VII

To demonstrate that harpin is produced by *E. amylovora* and not *E. coli* stimulated by the presence of pCPP430, the same techniques used for its isolation from *E. coli* DH5α (pCPP430) were used with *E. amylovora* Ea321, except that the cells were preincubated in a HR-inducing medium for 5 hrs before sonication. In addition, *E. coli* DH5α(pCPP9), which harbors the vector of pCPP430, was subjected to the same procedures as *E.coli* DH5α(pCPP430). A protein isolated with the same molecular weight as that isolated from Ea321, had HR-eliciting ability. Based on the relative intensity of the 44 kD band on SDS polyacrylamide gels, it was estimated that *E. amylovora* Ea321 produces, on a per cell basis, about one tenth the amount of harpin as does *E. coli* DH5α(pCPP430). The properties of the elicitor protein from *E. amylovora* Ea321 and *E. coli* DH5α(pCPP430) were identical. No protease-sensitive heat stable HR-eliciting activity associated with a 44 kD protein was seen in cell-free extracts taken from *E. coli* DH5α(pCPP9).

The properties of the *E. amylovora* harpin are consistent with several important physiological observations that were made following the discovery that bacteria can elicit the hypersensitive response. Infiltration of plant tissues with incompatible pathogens and inhibitors of bacterial protein or RNA synthesis prevent the hypersensitive response [see Phytopathology 72:1513 (1982)] indicating that de novo RNA and protein synthesis is required. When bacteria are infiltrated in dilute water agar, no hypersensitive response is elicited, suggesting that intimate contact between bacteria and plant cells is required. Pre-induced bacteria quickly lose HR-eliciting ability when infiltrated with translation or transcriptional inhibitors [see Science 245:1374 (1989)]. Further evidence that the elicitor is a component of the bacterial cell surface is found in observations that the elicitor is not diffusible in infiltrated plant tissue and that each introduced bacterium kills only one plant cell. As predicted by these observations, harpin is associated with the bacterial cell surface and appears unstable in nature because of its extreme sensitivity to proteolysis. Thus, harpin degradation may be important in regulating the development of the plant-bacterium interaction.

The nonpathogenic phenotype of hrp mutants suggest that harpin is also a primary determinant of pathogenicity in *E. amylovora*. The basis for the essential role for harpin in both compatible (host:disease) and incompatible (nonhost:hypersensitive response) interactions is not clear. Host range in some plant pathogenic bacteria has been shown to be controlled by avr genes that can confer cultivar-specific incompatibility to hrp+ pathogens. The biochemical activity of the avr gene products and the basis for their dependence on hrp genes for phenotypic expression is unknown, although avrb is regulated by hrp genes. Regulation of the production or accumulation of harpin may also be a determinative factor; the hrp gene cluster in *E. amylovora* is expressed about 10-fold lower in host tissue (pear) than in nonhost tissue (tobacco).

Although major disease determinants have been identified in plant pathogenic bacteria that cause either tumors or extensive tissue maceration (phytohormones and pectic enzymes, respectively), the molecular basis for pathogenicity among bacteria that cause delayed necrosis in a limited range of hosts is unknown. Among these bacteria are the economically important *Pseudomonas syringae* and *Xanthomonas campestris* pathovars. Toxins and plant cell wall degrading enzymes may increase the virulence of these pathogens, but the hrp genes are absolutely required for bacterial multiplication in host tissues and production of disease symptoms.

The conservation of the hrp genes [see Laby, R. J., Molecular studies on pathogenicity and virulence factors of *Erwinia amylovora*, M. S. Thesis, Cornell University (1991)] suggests that the *E. amylovora* harpin is the archetype of a broadly important class of plant bacterial disease determinants. Thus, disruption of harpin or of the proper balance of its production would be a novel approach to controlling the prevalent bacterial diseases of crop plants. The mode of action of harpin would also reveal the molecular bases for the hypersensitive response and for resistance of plants to a broad array of microbial pathogens.

The following example provides a description for the determination of the N-terminal amino acid sequence by which the gene encoding harpin was located.

EXAMPLE VIII

In order to locate the gene encoding harpin, named hrpN, the partial amino acid sequence of the harpin protein was determined. A sample of harpin (25 μg) purified by HPLC as in Example IV was used. A portion of the eluent from the reverse-phase chromatographic column corresponding to the peak eluting at 42.5 min was evaporated to near dryness in vacuo to eliminate the acetonitrile solvent. The fraction was then dissolved in TE buffer and submitted to the Protein Analysis Laboratory of the Cornell University Biotechnology Program with the request that the proportion of the various amino acids present in the protein, and the sequence of amino acids beginning from the N-terminus be determined.

The results of these analyses are shown, in the following table in which the amino acid composition from analysis of harpin differs only slightly from the amino acid composition deduced from the DNA sequence:

Amino Acid	% Deduced from DNA	% Deduced from Harpin
alanine	5.4	7.6
arginine	1.8	1.3
asparagine	7.0	
aspartic acid	5.7	14.2
cysteine	0	0
glutamine	6.5	
glutamic acid	2.1	9.3
glycine	22.0	22.0
histidine	0.8	<1.0
isoleucine	2.3	2.3
leucine	10.6	10.9
lysine	4.7	5.2
methionine	6.0	5.7
phenylalanine	1.6	2.0
proline	3.1	2.3
serine	9.6	8.9
threonine	6.2	5.2
tryptophan	0.5	—
tyrosine	1.0	<1.0
valine	2.8	2.2

The procedure used for the determination of amino acid composition included hydrolysis of the protein with 6N HCL followed by derivatation of the amino acid residues and resolution according to S.A. Cohen et al., 1984, American Laboratory p. 48.

The N-terminal amino acid sequence of harpin according to the present invention was determined according to the methods of Hunkapiller [see Methods Of Protein Microcharacterization; A Practical Handbook, ppg. 223–247, Humana Press, Clifton, N.J. 1986)] is as follows (SEQ ID No. 1):

```
Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr
                5                  10
Met Gln Ile
        15
```

The deduced amino acid sequence of harpin (including the N-terminal amino acid sequence given above) (SEQ ID No. 2) according to the present invention is:

```
Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser
 1               5                  10                  15

Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln
            20                  25                  30

Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn
        35                  40                  45

Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
    50                  55                  60

Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
65                  70                  75                  80

Gly Gly Gly Leu Gly Asn Gly Leu Gly Ser Gly Gly Leu Gly Glu
                85                  90                  95

Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr
            100                 105                 110

Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro
        115                 120                 125

Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser
    130                 135                 140

Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln
145                 150                 155                 160

Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly
                165                 170                 175

Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu
            180                 185                 190
```

-continued

```
Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly
        195                 200                 205

Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly
    210                 215                 220

Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Lgy Ser Ser Leu
225                 230                 235                 240

Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln
                245                 250                 255

Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln
            260                 265                 270

Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe
        275                 280                 285

Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met
    290                 295                 300

Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro
305                 310                 315                 320

Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser
                325                 330                 335

Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn
            340                 345                 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn
        355                 360                 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp
    370                 375                 380

Ala Met Met Ala Gly Asp Ala Ile Asn Ans Met Ala Leu Gly Lys Leu
385                 390                 395                 400

Gly Ala Ala
```

The partial amino acid sequence of harpin was utilized to construct an oligonucleotide probe with bases corresponding to those encoding the ninth to fifteenth amino acids of the N-terminus of harpin. Since several of these amino acids may have several nucleic acid codons, a 48-fold degenerate oligonucleotide was constructed according to standard procedures.

The identification of clones encoding harpin by hybridization with an oligonucleotide probe for harpin is described in the following example:

EXAMPLE IX

The structural gene encoding harpin was identified by hybridization of the oligonucleotide probe constructed in Example VIII with DNA of *Erwinia amylovora*. The specific DNA cloned in the hrp cluster of *E. amylovora* in cosmid pCPP430 was digested with the restriction enzyme BamHI and a separate portion was digested with the restriction enzyme HindIII. The DNA digests were electrophoresed in 0.7% agarose, stained with ethidium bromide, transferred to a nylon membrane (Immobilon) and hybridized with the oligonucleotide probe previously described, according to standard procedures. The probe was labelled with radioactive phosphorous using ^{32}p labelled GTP.

Following hybridization and exposure of the membranes to X-O-Mat X-ray film (Kodak) and development of the film, a 1.3 kb HindIII fragment gave the strongest hybridization signal in response to the probe. The fragment was subcloned in the pBluescript M13+ vector (Stratagene), and designated pCPP1084.

The production of anti-harpin antibodies according to the present invention is described in the following example:

EXAMPLE X

Antibodies were raised in rabbits in response to injection with harpin. Three injections of highly purified harpin (100, 150 and 50 μg, respectively) were made at 2–3 week intervals. The antiserum was harvested after 8 weeks, IgG was precipitated with ammonium sulfate, and preabsorbed with sonicated *E. coli* DH5α(pCPP9) lysate. The specificity of the antiserum was confirmed by reaction in western blots of harpin purified by HPLC as described in Example VII. No reaction was seen with pre-immune serum when western blots containing resolved CFEP from DH5α(pCPP430) were hybridized.

The description of hrpN in the T7 RNA polymerase/promoter expression system is described in the following example:

EXAMPLE XI

To confirm that the 1.3 kb HindIII fragment contains the entire hrpN gene, the plasmid pGpl-2 (Proc. Natl.Acad. Sci. U.S.A. 82:1074 (1985)] and pCPP1084, which contains the 1.3 kb HindIII fragment under the control of T7ϕ10 promoter, was transformed, into *E. coli* DH5α or Ea321. These two compatible plasmids constitute the T7 expression system. The cells containing both pGpl-1 and pCPP1084 were grown in LB with 100 μg/ml of ampicillin and 50 μg/ml of kanamycin at 30° C. Two hundred μl of cells at OD_{620}=0.5 were harvested and washed with 5 ml of M9

media [Sambrook, J., E. F. Fritsch, T. Maniatis, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor, (1989)]. Finally, the cells were resuspended in 1.0 ml of M9 medium supplemented with 0.01% of 18 amino acids (no cystidine or methionine). Cells were grown with shaking (200 rpm) at 30° C. for 1 hr then shifted to 42° C. for 10 min. Rifmapicin (Sigma R3501 20 mg/ml stock solution in methanol) was added to final concentration of 200 μg/ml. Cells were incubated at 42° C. for 10 additional minutes and then shifted to 30° C. and incubated for an additional 1 hour. Cells were pulsed with 10 μCi of 35_S methioine for 5 min at 30° C. The cells were centrifuged and resuspended in 50 μl of "cracking buffer" (60 mM Tris-HCl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue). The samples were heated at 100° C. for 3 min and 20 μl were placed on a 10% SDS PAGE gel. After electrophoresis at 15 mA for 2.0 hr in a Mighty Small™ apparatus (according to instruction of Hoefer Scientific Instruments), the gel was dried and exposed to X-ray film for 2 hrs at room temperature. A single 44 kD band, which corresponded in molecular size to harpin, was observed from both the *E. coli* DH5α and Ea321 constructs contains the entire open reading frame that encodes the 44 kD harpin protein.

The nucleic acid sequence of the hrpN gene according to the present invention was determined according to the following example.

EXAMPLE XII

DNA sequencing analysis was performed by the dideoxy-chain termination method (Sanger 1977, PNAS 74:5643–5667). The sequences were verified from both strands by using either the universal primer or the T3 primer. The subclones generated by KpnI and PtuI from the 1.3 kb HindIII fragment were used directly as templates for sequencing. The nucleotide sequence of hrpN was submitted to Genbank and assigned accession number M92994. The nucleotide sequence (SEQ ID No. 3) is shown below.

```
AAGCTTCGGC ATGGCACGTT TGACCGTTGG GTCGGCAGGG TACGTTTGAA TTATTCATAA   60

GAAGAATACG TTATGAGTCT GAATACAAGT GGGCTGGGAG CGTCAACGAT GCAAATTTCT  120

ATCGGCGGTG CGGCCGGAAA TAACGGGTTG CTGGGTACCA GTCGCCAGAA TGCTGGGTTG  180

GGTGGCAATT CTGCACTGGG GCTGGCGGC GGTAATCAAA ATGATACCGT CAATCAGCTG  240

GCTGGCTTAC TCACCGGCAT GATGATGATG ATGAGCATGA TGGGCGGTGG TGGGCTGATG  300

GGCGGTGGCT TAGCGGTGG CTTAGGTAAT GGCTTGGGTG GCTCAGGTGG CCTGGCCGAA  360

GGACTGTCGA ACGGCTGAA CGATATGTTA GGCGGTTCGC TGAACACGCT GGGCTCGAAA  420

GGCGGCAACA ATACCACTTC AACAACAAAT TCCCCGCTGG ACCAGCGCT GGGTATTAAC  480

TCAACGTCCC AAAACGACGA TTCCACCTCC GGCACAGATT CCACCTCAGA CTCCAGCGAC  540

CCGATGCAGC AGCTGCTGAA GATGTTCAGC GAGATAATGC AAAGCCTGTT TGGTGATGGG  600

CAAGATGGCA CCCAGGGCAG TTCCTCTGGG GGCAAGCAGC CGACCGAAGG CGAGCAGAAC  660

GCCTATAAAA AAGGAGTCAC TGATGCGCTG TCGGCCTGA TGGGTAATGG TCTGAGCCAG  720

CTCCTGGCA ACGGGGGACT GGGAGGTGGT CAGGCCGTA ATGCTGGCAC GGGTCTTGAC  780

GGTTCGTCGC TGGGCGCAA AGGGCTGCAA AACCTGAGCG GGCCGGTGGA CTACCAGCAG  840

TTAGGTAACG CCGTGGGTAC CGGTATCGGT ATGAAAGCGG GCATTCAGGC GCTGAATGAT  900

ATCGGTACGC ACAGGCACAG TTCAACCGBT TCTTTCGTCA ATAAAGCGA TCGGCGATG  960

GCGAAGCAAA TCGGTCAGTE CATGGACCAG TATCCTGAGG TGTTTGGCAA GCCGCAGTAC 1020

CAGAAAGGCC CGGGTCAGGA GGTGAAAACC GATGACAAAT CATGGGCAAA AGCACTCAGC 1080

AAGCCAGATG ACGACGGAAT GACACCAGCC AGTATGGAGC AGTTCAACAA AGCCAAGGGC 1140

ATGATCAAAA GGCCCATGGC GGGTGATACC GGCAACGGCA ACCTGCAGGC ACGCGGTGCC 1200

GGTGGTTCTT CGCTGGGTAT TGATGCCATG ATGGCCGGTG ATGCCATTAA CAATAIGGCA 1260

CTTGGCAAGC TGGGCGCGC TTAAGCTT                                      1288
```

The 44 kD band expressed from this system was also reacted with anti-harpin antibody raised in rabbit (Example X). This experiment demonstrated that the 1.3 kb HindIII fragment

In this sequence, the open reading frame (including the stop codon TGA) which is expressed to provide the amino acid sequence (SEQ ID No. 4) for to harpin is as follows:

```
ATGAGTCTGA ATACAAGTGG GCTGGGAGCG TCAACGATGC AAATTTCTAT CGGCGGTGCG  60

GGCGGAAATA ACGGGTTGCT GGGTACCAGT CGCCAGAATG CTGGGTTGGC TGGCAATTCT 120

GCACTGGGGC TGGGCGGCGG TAATCAAAAT GATACCGTCA ATCAGCTGGC TGGCTTACTC 180

ACCGGCATGA TGATGATGAT GAGCATGATG GCGGTGGTG GGCTGATGGG CGGTGGCTTA 240

GGCGGTGGCT TAGGTAATGG CTTGGGTGGC TCAGGTGGCC TGGGCGAAGG ACTGTCGAAC 300

GCGCTGAACG ATATGTTAGG CGGTTCGCTG AACACGCTGG GCTCGAAAGG CGGCAACAAT 360

ACCACTTCAA CAACAAATTC CCGCTGGAC CAGGCGCTGG GTATTAACTC AACGTCCCAA 420

AACGACGATT CCACCTCCGG CACAGATTCC ACCTCAGACT CCAGCGACCC GATGCAGCAG 480

CTGCTGAAGA TGTTCAGCGA GATAATGCAA AGCCTGTTTG GTGATGGCA AGATGGCACC 540

CAGGGCAGTT CCTCTGGGGG CAAGCAGCCG ACCGAAGGCG AGCAGAACGC CTATAAAAAA 600

GGAGTCACTG ATGCGCTGTC GGGCCTGATG GGTAATGGTC TGAGCCAGCT CCTTGGCAAC 660

GGGGGACTGG GAGGTGGTCA GGGCGGTAAT GCTGGCACGG GTCTTGACGG TTCGTCGCTG 720

GGCGGCAAAG GGCTGCAAAA CCTGAGCGGG CCGGTGGACT ACCAGCAGTT AGGTAACGCC 780

GTGGGTACCG GTATCGGTAT GAAAGCGGGC ATTCAGGCGC TGAATGATAT CGGTACGCAC 840

AGGCACAGTT CAACCCGTTC TTTCGTCAAT AAAGGCGATC GGGCGATGGC GAAGGAAATC 900

GGTCAGTTCA TGGACCAGTA TCCTGAGGTG TTTGGCAAGC CGCAGTACCA GAAAGGCCCG 960

GGTCAGGAGG TGAAAACCGA TGACAAATCA TGGGCAAAAG CACTGAGCAA GCCAGATGAC 1020

GACGGAATGA CACCAGCCAG TATCGAGCAG TTCAACAAAG CCAAGGGCAT GATCAAAAGC 1080

CCCATGGCGG GTGATACCGG CAACGGCAAC CTGCAGGCAC GCGGTGCCGG TGGTTCTTCG 1140

CTGGGTATTG ATGCCATGAT GGCCGGTGAT GCCATTAACA ATATGGCACT TGGCAAGCTG 1200

GGCGCGGCT                                                        1209
```

The over expression of the hrpN gene to produce large quantities of harpin is depicted in the following example:

EXAMPLE XIII

A new plasmid, designated pCPP50, was constructed especially for high expression of harpin as follows:

The expression vector pINIII^{113}-A2 [see Bio/Technology, pp 81-85 (January 1984)] was modified. It was digested with the restriction endonuclease XbaI and IIindIII which resulted in two fragments. The smaller DNA fragment was discarded and replaced with a portion of the pBluescript SK$^-$ polylinker (XbaI to HindIII). These manipulations removed the ribosome-binding site and initiation codon (ATG) from pINIII^{113}-A2 and replaced them with several useful cloning sites (XbaI, SpeI, BamHI, SmaI, PstI, EcoRV, HindIII, BamHI). The resulting vector (pCPP50) was used in conjunction with the hrpN gene to facilitate super-production of harpin by *E. coli*.

Plasmid pCPP1084, containing hrpN (Example VII) was digested with the restriction endonuclease HindIII. The 1.3 kb HindIII DNA fragment was purified from an agarose gel, and ligated into pCPP50 which had also been digested with HindIII and treated with alkaline phosphoratase. The DNA was transformed into *E. coli* DH5α. Several transformants were screened on an SDS-Polyacrylamide gel for production of a protein corresponding to the known mobility of harpin. One clone, designated pCPP2139, produced large quantities of harpin.

Large quantities of harpin were produced in *E. coli* DH5α(pCPP2139) according to the following procedure: *E. coli* DH5α(pCPP2139) was grown in M9 minimal medium supplemented with 5 g/l casamino acids and 40 mg/l thiamine. The bacteria were grown for an additional 20 hours at 37° C. Harpin was isolated from the bacteria according to Example III.

Harpin produced by *E. coli* DH5α(pCPP2139) was active in tobacco leaf assays and it had the same molecular weight on SDS-polyacrylamide gels and reacted with anti-harpin antiserum (Example X) as harpin produced by *E. coli* DH5α(pCPP430).

In dilution point tobacco leaf assays, CFEP produced from *E. coli* DH5α(pCPP2139) had detectable activity at a 1:150 dilution. *E. coli* DH5α(pCPP430) had detectable activity only to a 1:10 dilution. Thus, *E. coli* DH5α (pCPP2139) produced at least 15 times as much harpin as *E. coli* DH5α(pCPP430). The results referred to are tabulated in the following table.

TABLE 2

CFEP from	Dilutions					
E. coli strain	1:10	1:20	1:50	1:100	1:150	1:200
DH5α(pCPP2139)	+	+	+	+	+	−
DH5α(PCPP430)	−	−	−	−	−	−

+ = a positive reaction, collapse of tobacco tissue as in the hypersensitive response;
− = a negative reaction, no collapse of tobacco leaf tissue

Similar conclusions were drawn by examination of SDS-polyacrylamide gels containing harpin preparations from the two constructions.

In addition to determining hrpN in *E. amylovora*, and because harpin is believed to be the archetype for a family of proteinaceous HR elicitors that are produced by many different phytopathogenic bacteria, the identification of hrpN homologs was also searched out in *Erwinia chrysanthemi* and *Erwinia stewartii* according to the following protocol.

EXAMPLE XIV

The 1.3 kb HindIII DNA fragment from pCPP1084, containing hrpN, was used as a radioactive probe against 18 cosmids previously shown to contain hrp genes from *E. chrysanthemi* strain AC4150. One cosmid, pCPP2157, hybridized strongly with the HrpN clone under high stringency conditions (washes done in 0.4 ×SSC, 0.2% SDS, 65° C.). The cosmid was used in further analyses. An 800 bp Cla1 fragment from pCPP2157, which hybridized with the HrpN probe, was cloned into pBluescript SK− to give pCPP2140. Initial DNA sequencing (using Sequenase version 2.0 kit, U.S. Biochemicals) of one end of the 800 bp Cla1 fragment showed a region of 224 nucleotides with 72% nucleotide identity. Sequence comparison was done with FASTA., and the nucleotide sequence for *E. chrysanthemi* corresponding to *E. amylovora* hrpN (best-fit) from nucleotide 1005 to 1223 indicates a 72% identity. The *E. chrysanthemi* sequence (SEQ ID No. 5) is given below.

```
CGGTAAACCG GATACCAGAA AGATGGCTGG AGTTCGCCAG AAGACGGACG 50
ACAAATCCTG GGCTAAAGCG CTCAGTAAAC CGGATGATGA CGGTATCACC 100
GGTCTGCCAG CATGGACAAA TTCCGTCAGG CGATGGGTAT GATCAAAAGC 150
GCGGTGCCGG CTGACTACCG CATACCAAC CTGAATCTGC GTGGCGCGGG 200
CGGTGCATCG CTGGGTATCG AT                               222
```

Using a similar protocol, the 1.3 kb HInd III DNA fragment from pCPP1084 was used to probe a DNA of *E. stewartii*. Genomic DNA of strain DC283 and DNA of the cosmid clone pES411 [see Coplin et al., Mol. Plant-Microbe Interactions. 5:266–268 (1992)] were hydrolysed with Hind III, electrophoresed and hybridized. A 1.8 kb Hind III fragment from both DNA preparations hybridized with the probe. These results indicate that hrpN of *E. amylovora* shares homology with a hrpN-like gene of *E. stewartii*.

The effect of two means of inactivation, according to the present invention, of harpin on disease severity in plants is described below.

EXAMPLE XV

Inactivation of harpin by reaction of *E. amylovora* cells with an antiserum specific for harpin (Example X) or a protease that degrades harpin (Example VII) resulted in a reduction in disease of pear caused by *E. amylovora*. Immature pear fruit, harvested when the fruit were 3–4 cm in diameter were surface-disinfested, cut in half lengthwise and placed on moistened paper towels. Wells were cut in the cheeks of fruit with a number 1 cork borer (see Beer, S. V. Methods in Phytobacteriology, pp 372–375 (1990) Klement, Z., Rudolf, K, and Sands, D. eds). One ml of a culture of Ea321 (2×10^8 cfu/ml) was mixed with 50 μl and 100 μl of a 1:25 dilution of anti-harpin antisera (Example X), and after 5 minutes, 50 μl of the mixture was deposited in the well of each pear fruit. Similarly, suspensions of Ea321 were mixed with protease before deposit in the wells in the pear fruit. The pears were incubated at 27° C. and observed daily for 3 days. Controls consisted of cells not treated and cells mixed with pre-immune serum taken from the same rabbit. The results are tabulated below:

Treatment	Infection*
Ea321	8/8
Ea321 + Protease (100 μg/ml)	6/8
Ea321 + Protease (200 μg/ml)	5/8
Ea321 + Antiserum (50 μl/ml)	5/8
Ea321 + Antiserum (100 μl/ml)	5/8
Ea321 + Preimmune Serum (100 μl/ml)	8/8

*Number of treated pear halves (out of 8) showing ooze at cut ends 64 hours after inoculation with 50 μl containing 1×10^6 cfu of Ea321 treated as indicated.

Treatment of *E. amylovora* with either protease or harpin-specific antiserum reduced the number of pear fruits that became infected. Treatment with preimmune (normal) serum had no effect on the development of disease. The above-described test of the effect of two treatments that affect harpin without affecting the vitality or growth of *E. amylovora* was particularly harsh. Only the harpin present on the treated cells could be affected because the antiserum or enzyme could not be present to react with harpin on the progeny from the treated cells. Under conditions envisioned for practical use according to the present invention, anti-harpin antibodies would be produced by plants transformed with genes encoding anti-harpin antibodies or protease, and these in turn would inhibit or lessen the disease severity of the plant exposed to the elicitor. Also, in nature, treatment of blooming apple or pear trees with protease or anti-harpin antibodies is likely to result in greater reductions in fire blight because infections generally are initiated by a small number of cells, as opposed to about 10^8, as was used in the above example.

Thus, to summarize the present invention, there is strong evidence that harpin is the archetype for proteinaceous factors that enable plant pathogenic bacteria (and possibly other pathogenic microorganisms) to elicit either the hypersensitive response in nonhosts or to promote disease in hosts. To begin with, strains of the three genera Erwinia, Pseudomonas, and Xanthomonas elicit a very similar (visually and physiologically) hypersensitive response when infiltrated into leaves of their respective non-host plants. This relationship has been documented almost since the discovery of the hypersensitive response elicited by bacteria in 1963. In addition, the genes required for the elicitation of the HR by strains of all three genera of bacteria (referred to similarly, as hrp genes) are also those required for both pathogenicity to host plants and for elicitation of the hypersensitive response in non-host plants.

The relationship between hrp genes among phytopathogenic bacteria has been documented in studies by Laby and Beer [Molecular Plant Microbe Interactions 5:(1992); R. J. Laby, Molecular studies on pathogenicity and virulence factors of *Erwinia amylovora*, M. S. Thesis, Cornell University, Ithaca, N.Y. 1991]. They showed conclusively

relationships, at the DNA level, between the hrp gene cluster of *E. amylovora* and the hrp gene cluster of Pseudomonas syringae, as well as the relationship between the hrp gene cluster of *E. amylovora* and the wts (water soaking) gene cluster of *E. stewartii*. Other workers have demonstrated a striking relationship among the hrp genes of various *P. syringae* pathovars (strains of *P. syringae* pathogenic to specific and different plants). Still other researchers have demonstrated a close relationship between hrp genes of strains of *Xanthomonas campestris* and *P. solanacearum*. Thus, there is overwhelming evidence for conserved DNA among plant pathogenic bacteria of several genera that cause disease of a multitude of plants.

The significant similarity in DNA sequence between the hrpN gene of *E. amylovora* and a homologous gene of *E. chrysanthemi*, according to the present invention, has also been shown . In addition, we have observed strong hybridization between hrpN and genomic DNA of *E. stewartii*, a serious pathogen of maize. More specifically, hybridization between hrpN and a specific 1.8 kb Hind III fragment of the wts gene cluster was observed. This indicates that the other two species of Erwinia examined to date have hrpN homologs. Thus, significant similarity in the hrpN-like gene products (protein) according to the present invention can be expected.

In addition, many of the hrp genes of *E. amylovora* appear to be involved in the secretion of cell-surface exposition of harpin, based on the pleiotype of mutations in those genes. One gene of the hrp gene cluster of *Pseudomonas syringae*, which hybridizes with a portion of the hrp gene cluster of *E. amylovora*, encodes a protein with a high amino acid similarity with proteins involved in secretion in various Gram-negative bacteria.

Thus, the known similarities of hrp genes of Pseudomonas, Xanthomonas, and Erwinia provide a firm basis to suspect that the HR elicitors produced by strains of the three genera are likely to be similar in amino acid sequence or at least in general characteristics (protein) and function.

The uses to which the various aspects and portions of the present invention may be put to are many and varied. For example, hrpN mutants may be used to identify, by complementation, genes from other plant pathogenic organisms (e.g., bacteria, fungi, nematodes) that encode proteins that function similarly to harpin. Although such proteins may have substantially different primary structures (and therefore would be difficult to detect by DNA hybridization techniques), these proteins should restore the ability to elicit the HR to either *E. amylovora* or *E. coli* cells carrying a hrp cluster that was functional, except for the hrpN gene.

Another use within the scope of the present invention is to use harpin and/or harpin-producing strains to identify in plants harpin receptors and/or their interactants in signal transduction pathways and clone their encoding genes. Thus, this would allow one to exploit the potential of harpin to function (depending upon the plant) as a pathogenicity factor or as an elicitor of defense reactions to manipulate the structure or expression of plant genes (s) encoding harpin receptor(s) for the purpose of producing genetically engineered plants with improved resistance to plant pathogens.

Still another use of harpin within the scope of the present invention would be as a potentiator of secondary metabolite production in plants grown either naturally or in tissue culture.

Still another use would be the fusion of the gene encoding harpin to specific promoters of plant genes to develop specific transgenic plants. When the plant gene is "turned on", harpin would be expressed and the plant cell killed. Some appropriate plant gene promoters and their projected uses include genes involved in pollen development (resulting in the development of male sterile plants); genes that are expressed in response to infection by fungi, e.g. genes encoding phenylalanine ammonia lyase and chalcone synthase the plant cell would be killed thereby limiting the progress of the fungus and making the plant resistant to fungal diseases); and genes involved in the development of senescence (to facilitate harvest, expression of hrp genes would result in defoliation).

Still another use of harpin within the scope of the present invention would be the use of harpin as a "target molecule" with which chemical compounds would be designed to react and thereby inactivate the bacterial harpin, which, because it is essential for disease, would provide a specific bacteriacide target.

A listing of the nucleotide and amino acids described in the present application are as follows:

Thus while we have illustrated and described the preferred embodiment of our invention, it is to be understood that this invention is capable of variation and modification, and we therefore do not wish to be limited to the precise terms set forth, but desire to avail ourselves of such changes and alterations which may be made for adapting the invention to various usages and conditions. Such variations and modifications, for example, would include the substitution of structurally similar sequences, for both the elicitor and hrpN genes provided herein (whether derived from natural sources or synthetically manufactured), which function to yield substantially similar activities to those specifically described above. Thus, changes in sequence by the substitution, deletion, insertion or addition of nucleic acids (in the DNA sequences) or amino acids (in the peptide sequences) which do not substantially alter the function of those sequences specifically described above are deemed to be within the scope of the present invention. Accordingly, such changes and alterations are properly intended to be within the full range of equivalents, and therefore within the purview of the following claims.

Having thus described our invention and the manner and a process of making and using it in such full, clear, concise and exact terms so as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same;

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 5

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 403 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser
1               5                  10                  15

Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln
            20                  25                  30

Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn
        35                  40                  45

Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
    50                  55                  60

Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
65                  70                  75                  80

Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu
                85                  90                  95

Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr
            100                 105                 110

Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro
        115                 120                 125

Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser
    130                 135                 140

Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln
145                 150                 155                 160

Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly
                165                 170                 175

Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu
            180                 185                 190

Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly
        195                 200                 205

Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly
    210                 215                 220

Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu
```

-continued

```
225                 230                 235                 240

Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln
                245                 250                 255

Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln
            260                 265                 270

Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe
        275                 280                 285

Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met
    290                 295                 300

Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro
305                 310                 315                 320

Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser
                325                 330                 335

Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn
            340                 345                 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn
        355                 360                 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp
    370                 375                 380

Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu
385                 390                 395                 400

Gly Ala Ala
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1288 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
AAGCTTCGGC ATGGCACGTT TGACCGTTGG GTCGGCAGGG TACGTTTGAA TTATTCATAA     60

GAGGAATACG TTATGAGTCT GAATACAAGT GGGCTGGGAG CGTCAACGAT GCAAATTTCT    120

ATCGGCGGTG CGGGCGGAAA TAACGGGTTG CTGGGTACCA GTCGCCAGAA TGCTGGGTTG    180

GGTGGCAATT CTGCACTGGG GCTGGGCGGC GGTAATCAAA ATGATACCGT CAATCAGCTG    240

GCTGGCTTAC TCACCGGCAT GATGATGATG ATGAGCATGA TGGGCGGTGG TGGGCTGATG    300

GGCGGTGGCT TAGGCGGTGG CTTAGGTAAT GGCTTGGGTG GCTCAGGTGG CCTGGGCGAA    360

GGACTGTCGA ACGCGCTGAA CGATATGTTA GGCGGTTCGC TGAACACGCT GGGCTCGAAA    420

GGCGGCAACA ATACCACTTC AACAACAAAT TCCCCGCTGG ACCAGGCGCT GGGTATTAAC    480

TCAACGTCCC AAAACGACGA TTCCACCTCC GGCACAGATT CCACCTCAGA CTCCAGCGAC    540

CCGATGCAGC AGCTGCTGAA GATGTTCAGC GAGATAATGC AAAGCCTGTT TGGTGATGGG    600

CAAGATGGCA CCCAGGGCAG TTCCTCTGGG GGCAAGCAGC CGACCGAAGG CGAGCAGAAC    660

GCCTATAAAA AAGGAGTCAC TGATGCGCTG TCGGGCCTGA TGGGTAATGG TCTGAGCCAG    720

CTCCTTGGCA ACGGGGACT GGCAGGTGGT CAGGGCGGTA ATGCTGGCAC GGGTCTTGAC    780

GGTTCGTCGC TGGGCGGCAA AGGGCTGCAA AACCTGAGCG GCCGGTGGA CTACCAGCAG    840

TTAGGTAACG CCGTGGGTAC CGGTATCGGT ATGAAAGCGG GCATTCAGGC GCTGAATGAT    900

ATCGGTACGC ACAGGCACAG TTCAACCCGT TCTTTCGTCA ATAAAGCGA TCGGGCGATG    960
```

-continued

GCGAAGGAAA TCGGTCAGTT CATGGACCAG TATCCTGAGG TGTTTGGCAA GCCGCAGTAC 1020

CAGAAAGGCC CGGGTCAGGA GGTGAAAACC GATGACAAAT CATGGGCAAA AGCACTGAGC 1080

AAGCCAGATG ACGACGGAAT GACACCAGCC AGTATGGAGC AGTTCAACAA AGCCAAGGGC 1140

ATGATCAAAA GGCCCATGGC GGGTGATACC GGCAACGGCA ACCTGCAGGC ACGCGGTGCC 1200

GGTGGTTCTT CGCTGGGTAT TGATGCCATG ATGGCCGGTG ATGCCATTAA CAATAWGGCA 1260

CTTGGCAAGC TGGGCGGGC TTAAGCTT 1298

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1209 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATGAGTCTGA ATACAAGTGG GCTGGGAGCG TCAACGATGC AAATTTCTAT CGGCGGTGCG 60

GGCGGAAATA ACGGGTTGCT GGGTACCAGT CGCCAGAATG CTGGGTTGGG TGGCAATTCT 120

GCACTGGGGC TGGGCGGCGG TAATCAAAAT GATACCGTCA ATCAGCTGGC TGGCTTACTC 180

ACCGGCATGA TGATGATGAT GAGCATGATG GGCGCTGGTG GGCTGATGGG CGGTGGCTTA 240

GGCGGTGGCT TAGGTAATGG CTTGGGTGGC TCAGGTGGCC TGGGCGAAGG ACTGTCGAAC 300

GCGCTGAACG ATATTGTTAGG CGGTTCGCTG AACACGCTGG GCTCGAAAGG CGGCAACAAT 360

ACCACTTCAA CAACAAATTC CCGCCTGGAC CAGGCGCTGG GTATTAACTC AACGTCCCAA 420

AACGACGATT CCACCTCCGG CACAGATTCC ACCTCAGACT CCAGCGACCC GATGCAGCAG 480

CTGCTGAAGA TGTTCAGCGA GATAATGCAA AGCCTGTTTG GTGATGGGCA AGATGGCACC 540

CAGGGCAGTT CCTCTGGGGG CAAGCAGCCG ACCGAAGGCG AGCAGAACGC CTATAAAAAA 600

GGAGTCACTG ATGCGCTGTC GGGCCTGATG GGTAATGGTC TGAGCCAGCT CCTTGGCAAC 660

GGGGGACTGG GAGGTGGTCA GGGCGGTAAT GCTGGCACGG GTCTTGACGG TTCGTCGCTG 720

GGCGGCAAAG GGCTGCAAAA CCTGAGCGGG CCGGTGGACT ACCAGCAGTT AGGTAACGCC 780

GTGGGTACCG GTATCGGTAT GAAAGCGGGC ATTCAGGCGC TGAATGATAT CGGTACGCAC 840

AGGCACAGTT CAACCCGTTC TTTCGTCAAT AAAGCGATC GGGCGATGGC GAAGGAAATC 900

GGTCAGTTCA TGGACCAGTA TCCTGAGGTG TTTGGCAAGC CGCAGTACCA GAAAGGCCCG 960

GGTCAGGAGG TGAAAACCGA TGACAAATCA TGGGCAAAAG CACTGAGCAA GCCAGATGAC 1020

GACGGAATGA CACCAGCCAG TATGGAGCAG TTCAACAAAG CCAAGGGCAT GATCAAAAGG 1080

CCCATGGCGG GTGATACCGG CAACGGCAAC CTGCAGGCAC GCGGTGCCGG TGGTTCTTCG 1140

CTGGGTATTG ATGCCATGAT GGCCGGTGAT GCCATTAACA ATATGGCACT TGGCAAGCTG 1200

GCCGCGGCT 1209

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 222 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

-continued

```
CGGTAAACCG GATACCAGAA AGATGGCTGG AGTTCGCCAG AAGACGGACG ACAAATCCTG    60

GGCTAAAGCG CTGAGTAAAC CGGATGATGA CGGTATGACC GGTCTGCCAG CATGGACAAA   120

TTCCGTCAGG CGATGGGTAT GATCAAAAGC GCGGTGGCGG GTGATACCGG CAATACCAAC   180

CTGAATCTGC GTGGCGCGGG CGGTGCATCG CTGGGTATCG AT                      222
```

We claim:

1. *Escherichia coli* DH5α(pCPP1084) deposited under ATCC Accession No. 69021.

2. An isolated DNA molecule encoding a protein which elicits a hypersensitive response in plants, wherein the protein is encoded by a nucleic acid sequence which is complementary to a nucleic acid sequence which hybridizes to the nucleic acid of SEQ ID. No. 4 under stringent conditions of 0.4×SSC, 0.2% SDS washing at 65° C.

3. An isolated DNA molecule according to claim **2**, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID. No. 4.

4. An isolated DNA molecule according to claim **2**, wherein said protein has no cysteine.

5. An isolated DNA molecule according to claim **2**, wherein said protein has a molecular weight of 44 kDa on an SDS polyacrylamide gel.

6. An expression system containing the DNA molecule according to claim **2**, wherein the DNA molecule is heterologous to the expression system.

7. An expression system according to claim **6**, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID. No. 4.

8. An expression system according to claim **6**, wherein said protein has no cysteine.

9. An expression system according to claim **6**, wherein said protein has a molecular weight of 44 kDa on an SDS polyacrylamide gel.

10. A host cell containing the DNA molecule according to claim **2**, wherein the DNA molecule is heterologous to the host cell.

11. A host cell according to claim **10**, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID. No. 4.

12. A host cell according to claim **10**, wherein said protein has no cysteine.

13. A host cell according to claim **10**, wherein said protein has a molecular weight of 44 kDa on an SDS polyacrylamide gel.

14. A host cell according to claim **10**, wherein the DNA molecule is in an expression system.

15. A transgenic plant transformed with the isolated DNA molecule of claim **2**, wherein the DNA molecule is heterologous to the transgenic plant.

16. A transgenic plant according to claim **15**, wherein the DNA molecule comprises a nucleotide sequence of SEQ ID. No. 4.

17. An isolated DNA molecule according to claim **2**, wherein the hypersensitive response elicitor protein is from an Erwinia pathogen.

18. An isolated DNA molecule according to claim **17**, wherein the Erwinia pathogen is *Erwinia amylovora*.

19. An isolated DNA molecule according to claim **18**, wherein the protein comprises an amino acid sequence of SEQ ID. No. 2.

20. An isolated DNA molecule according to claim **17**, wherein the Erwinia pathogen is *Erwinia chrysanthemi*.

21. An isolated DNA molecule according to claim **17**, wherein the Erwinia pathogen is *Erwinia stewartii*.

22. An isolated DNA molecule according to claim **2**, wherein the protein is protease sensitive, contains no cysteine, and is heat stable at 100° C. for at least one minute.

* * * * *

Exhibit 6 - Tampakaki et al., *MPMI* 13:1366-1374 (2000)

MPMI Vol. 13, No. 12, 2000, pp. 1366–1374. Publication no. M-2000-0925-02R.

Elicitation of Hypersensitive Cell Death by Extracellularly Targeted $HrpZ_{Psph}$ Produced In Planta

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The ability of the *Pseudomonas syringae* pv. *phaseolicola* harpin ($HrpZ_{Psph}$) to elicit hypersensitive response was investigated in three *Nicotiana* genotypes. The $hrpZ_{Psph}$ gene was placed under chemical regulation (tetracycline induction) in TetR" *Nicotiana tabacum* cv. Wisconsin 38 (W38) or was transiently expressed in *N. benthamiana* following infection with a PVX-derived vector and in three *Nicotiana* genotypes by agroinfiltration. The constructs were designed to express either the canonical form of harpin ($HrpZ_{Psph}$) or an N-terminally extended version of the protein carrying the signal peptide portion of the tobacco pathogenesis-related protein PR1a ($SP\text{-}HrpZ_{Psph}$). Stable transformants of *N. tabacum* cv. W38 did not develop necrosis upon induction with tetracycline, probably as a result of insufficient harpin accumulation. In contrast, *N. benthamiana* plants infected with the PVX constructs produced high concentrations of harpin in biologically active form, but only those expressing the secretable form of harpin developed necrotic symptoms. These symptoms were less severe than those caused by PVX::*avrPto*; however, they were accompanied by induction of *hsr203J*, a hypersensitive response-specific gene transcript. These results suggest that the plant cellular receptor(s) for harpin is extracellular.

Additional keywords: bacterial hypersensitive response elicitor, bean halo blight pathogen, *hrp-hrc* genes, type III secreted protein.

The interaction of plant-pathogenic bacteria with resistant host cultivars or nonhost plants often leads to a rapid, localized defense response termed hypersensitive response (HR), during which cells immediately surrounding the site of infection rapidly die (Klement et al. 1964). There is accumulating evidence that HR is a programmed cell death process (Greenberg et al. 1994; Wang et al. 1996) that is triggered following specific recognition between plant and pathogen proteins that are "functionally correspondent" and may interact physically at some early stage of the pathogen-plant encounter (Flor 1971). Following the initial discovery by Lindgren et al. (1986), it has been established that the elicitation of HR by a broad range of plant-pathogenic bacteria, including *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Ralstonia* spp., requires the functions of a suite of genes originally named *hrp* (HR and pathogenicity) (Bonas 1994) and more recently known as *hrp-hrc* (HR-conserved) (Bogdanove et al. 1996). These genes encode components of a novel protein secretion pathway designated type III secretion system (TTSS) (Van Gijsegem et al. 1993). This pathway is similar to those used by mammalian pathogens to secrete proteinaceous virulence factors such as Yops, Sips, and Ipas, some of which are delivered by the bacterial-secretion apparatus inside the mammalian host cell (Hueck 1998).

In plant pathogens the type III pathway is used to deliver proteins from the bacterial cytoplasm either to the culture medium or into the host cell cytosol. Some of these bacterial proteins can stimulate or interfere with host cellular processes (effectors), whereas others are involved in the secretion process, its regulation, or the translocation of the effectors through the host cell membrane. Effectors delivered by TTSS in plant-pathogenic bacteria elicit HR or contribute to pathogen virulence (Leach and White 1996). Effectors such as Avr proteins and harpins are intrinsically able to trigger HR in the absence of other bacterial proteins. Only the harpins and PopA1, however, elicit HR when supplied to the plant in purified form at relatively high concentrations (Arlat et al. 1994; He et al. 1993; Wei et al. 1992). In contrast, Avr proteins elicit HR only when they are expressed endogenously in the plant cells (Gopalan et al. 1996; Leister et al. 1996; McNellis et al. 1998; Scofield et al. 1996; Van den Ackerveken et al. 1996). These effectors all require the bacterial type III pathway to exit from the bacterial cell (e.g., harpins and PopA1) or to reach their appropriate destination in the host cell (e.g., Avr proteins). These findings, coupled with the fact that Avr proteins are generally not secreted by the bacteria in culture, led to the hypothesis that these proteins are delivered directly inside the plant cells via the type III secretion pathway and initiate cell death by interacting with cognate receptors. This hypothesis is in line with the intracellular nature of most "matching" R gene products (putative receptors) (De Wit 1997; Scofield et al. 1996; Tang et al. 1996). The intracellular delivery model is also in line with the ability of the type III system of mammal-

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ian pathogens to deliver virulence proteins inside the host cell (Hueck 1998).

The role of harpins in compatible and incompatible interactions is still enigmatic. Inferences about their site of action have been made from two types of studies. The HR induced by harpin from *Erwinia amylovora* ($HrpN_{Ea}$) and *Pseudomonas syringae* pv. *syringae* ($HrpZ_{Psy}$) is prevented by inhibitors of calcium influx and ATPase activity in tobacco (He et al. 1993, Popham et al. 1995). The $HrpN_{Ea}$-induced membrane depolarization, extracellular alkalinization, and potassium efflux in tobacco suspension culture cells has indicated that harpin has a pronounced effect on the plasmalemma, affecting H^+-ATPase, ion channels, or membrane carriers (Pike et al. 1998, Popham et al. 1995). Moreover, Hoyos et al. (1996) reported that $HrpZ_{Psy}$ binds to the outer portion of the cell surface of tobacco suspension culture cells and causes K^+ efflux and extracellular alkalinization in tobacco cell suspension cultures but not in protoplasts. This work showed that $HrpZ_{Psy}$ is not detected in the cytoplasm of fixed, permeabilized, suspension-cultured cells but in the periphery of plant cells with intact walls, suggesting that $HrpZ_{Psy}$ possibly interacts with a component of the plant cell wall rather than the plasma membrane. These results do not necessarily rule out either possible interaction of HrpZ with the plasmalemma or internalization upon application to the extracellular medium of suspension cells due to the inherent limitations of the protoplast system and, possibly, to the low sensitivity of the immunofluorescence method.

To circumvent the limitations of these approaches and obtain further evidence concerning the mode and cellular site of harpin action, a fundamentally different approach was employed in the present study. Specifically, we investigated whether the HrpZ protein of *P. syringae* pv. *phaseolicola* ($HrpZ_{Pph}$) can elicit necrosis when expressed endogenously in plants. We expressed the protein in its canonical form and as a fusion with SP-PR1a, a plant protein signal peptide sequence. Our results showed that $HrpZ_{Pph}$ elicits HR only when it is produced in sufficient quantity by the plant and, importantly, in a secretable form.

RESULTS

Expression of *hrpZ*$_{Pph}$ in plants via *Agrobacterium*-mediated transformation.

Expecting that endogenously produced harpin may be lethal to the plant, we used the tetracycline inducible (Tet) expression vector system to achieve conditional expression of the *hrpZ*$_{Pph}$ gene. The *hrpZ*$_{Pph}$ coding frame was cloned in sense and antisense orientation in the pBin-Hyg-TX vector. To direct the $HrpZ_{Pph}$ protein to the plant cell exterior, the signal peptide (SP) sequence of the extracellular pathogenesis-related tobacco protein PR1a (SP_{Pr1a}) was fused in frame to the *hrpZ* coding sequence. Transgenic tobacco plants ($TetR^+$, *Nicotiana tabacum* cv. Wisconsin 38 [W38]) were transformed by *Agrobacterium*-mediated leaf disk transformation. Thirty hygromycin-resistant, independent transformants were obtained for each transforming plasmid. Analysis of the transformants by Northern hybridization and immunoblotting revealed an *hrpZ*$_{Pph}$-specific transcript and the $HrpZ_{Pph}$ protein after tetracycline treatment of leaves. The level of $HrpZ_{Pph}$ protein did not vary significantly among different transformants. Contrary to our expectations, the primary transformants (T_0) and selfed progeny (T_1) did not reveal necrotic or other types of symptoms of any form during several days of observation after administration of tetracycline either via the root system or by injection in leaf mesophyll panels. Similar results were obtained by agroinfiltration of three *Nicotiana* genotypes (*N. tabacum* cultivars Xanthi and W38 and *N. benthamiana*). In these plants both the canonical and the secretable form of $HrpZ_{Pph}$ were produced (not shown).

The failure of endogenously produced harpin to elicit necrotic symptoms could be a result of the protein not accumulating in sufficient quantity in the transgenic plants. Therefore, the amount of harpin produced in transgenic plants after tetracycline induction was quantified by immunoblotting and a rabbit polyclonal antibody prepared against $HrpZ_{Pph}$, which was purified from *Escherichia coli* and densitometry scanning of the bands (Fig. 1) with the National Institute of Health Image Program for Macintosh. Harpin accumulation was determined in total protein extracts from leaves after tetracycline induction from a reference plot that was based on known concentrations of purified protein. Two major immunoreactive

Fig. 1. Western blot analysis of $HrpZ_{Pph}$. Lane 1, Intercellular spaces of tobacco leaves after injection of *Pseudomonas syringae* pv. *phaseolicola*. The quantity corresponds to 0.2 cm^2 of leaf area. Lane 2, Leaves of *Nicotiana benthamiana* infected with PVX202::*hrpZ*$_{Pph}$ in 0.076 cm^2 of leaf area. Lane 3, Leaves of transformed plants $TetR^+$/$HrpZ^+$ 4 days after tetracycline induction in hydroponic culture. The quantity corresponds to 0.2 cm^2 of leaf area. P = purified $HrpZ_{Pph}$ from *Escherichia coli* (20 ng).

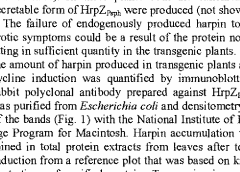

Fig. 2. Quantitative analysis of $HrpZ_{Pph}$. a, purified $HrpZ_{Pph}$ needed for hypersensitive response (HR) elicitation. b, $HrpZ_{Pph}$ produced in situ upon injection of *Pseudomonas syringae* pv. *phaseolicola* suspension into the intercellular spaces of tobacco leaf apoplast. c and d, $HrpZ_{Pph}$ produced in tobacco transgenic plants $TetR^+$/$HrpZ^+$ and $TetR^+$/SP-$HrpZ^+$ expressing the canonical and the secretable form of $HrpZ_{Pph}$, respectively, after 4 days of induction with 1 mg of tetracycline per l in hydroponic culture. e, $HrpZ_{Pph}$ accumulated in leaves of *Nicotiana benthamiana* 12 days after infection with PVX202::*hrpZ*$_{Pph}$. Results represent mean values of two independent experiments. In each experiment, three leaves from different plants with the same treatment were used. Vertical axis is logarithmic and shows the quantity of $HrpZ_{Pph}$ per square centimeter of leaf area. Intercellular space of well-expanded tobacco leaves was soaked with 10 $\mu l/cm^2$ of leaf area.

bands were observed in protein extracts from plants expressing the *hrpZ* gene. One corresponds to the full-length protein (upper band), and the other differs in apparent molecular mass by approximately 2 kDa. The origin of the lower band will be addressed later.

The quantity of harpin produced was compared with that produced by 10^7 CFU of *P. syringae* pv. *phaseolicola* per ml upon injection into the tobacco leaf apoplast and 100 μg of purified harpin per ml, the lowest concentrations able to elicit HR (Fig. 2). Harpin accumulation in the transformed plants (mass of protein/cm^2 of leaf) was 10-fold higher than the amount produced by bacteria in situ in standard HR tests. The bacteria, however, are most likely to deliver and/or specifically direct the elicitor to its correct cellular destination, possibly contributing other factors for HR development such as Avr proteins. In contrast, the transformed plants accumulated 50-fold less harpin than the quantity needed for HR elicitation when the protein is administered in purified form. The inability of harpin to elicit macroscopically visible HR in these plants may be attributed to insufficient quantity, improper delivery, or lack of biological activity of the protein when it is produced endogenously in the plant.

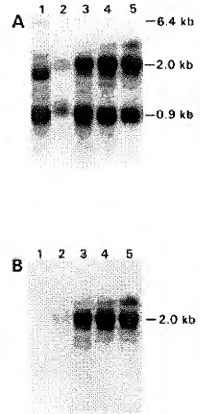

Fig. 3. Northern blot analysis of mRNA extracted from *Nicotiana benthamiana* plants after inoculation with PVX and PVX202::*hrpZ*$_{Psph}$. The membrane was probed sequentially with a riboprobe specific for the positive strand of PVX (**A**) and with a ^{32}P-labeled DNA probe of the entire *hrpZ*$_{Psph}$ sequence (**B**). Lanes: 1, total RNA extracted from leaves of PVX-infected plants 15 days after inoculation; 2 to 5, total RNA extracted from leaves of PVX202::*hrpZ*$_{Psph}$-infected plants at 8, 12, 16, and 20 days after inoculation, respectively. The position of various mRNA species are indicated on the left-hand margin. Autoradiographs were produced by exposure of panel A for 15 min, except lane 1, which was exposed for 1 h to detect the 6.4-kb transcript.

PVX-mediated expression of HrpZ$_{Psph}$ in *N. benthamiana*.

In an attempt to increase the intracellular accumulation of harpin, the *hrpZ*$_{Psph}$ gene initially was cloned into the potato virus X-derived vector pPVX202, which permits high-level expression of heterologous genes in solanaceous plants. DNA of the recombinant virus PVX202::*hrpZ*$_{Psph}$ was used to infect *N. benthamiana* leaves following the standard procedure (Sablowski et al. 1995). Control plants infected with PVX202 showed the characteristic chlorotic mosaic symptoms and leaf curling in the systemically infected leaves 12 to 15 days after infection. Plants infected with PVX202::*hrpZ*$_{Psph}$ produced symptoms similar to those of the control plants over the same time period and did not develop any form of necrosis. The HrpZ$_{Psph}$ protein was detected by Western blot in extracts from systemically infected leaves with PVX202::*hrpZ*$_{Psph}$ 8 to 9 days after inoculation. In the same period, the expected subgenomic viral transcripts were detected by Northern blot analysis by a riboprobe that was specific for the positive strand of PVX (Fig. 3). The quantity of harpin in these plants, quantified by densitometry scanning as described above, was substantially higher (100-fold) than in stably transformed *N. tabacum* cv. W38 TetR'/HrpZ'-expressing plants (Fig. 1). This amount was several-hundred-fold greater than that produced by bacteria injected into the leaf mesophyll at the lowest concentration needed to elicit confluent HR and roughly equal to the quantity of pure harpin able to elicit the reaction (Fig. 2).

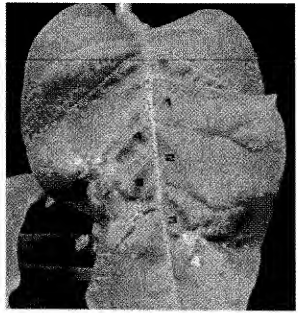

Fig. 4. Tobacco leaf showing the necrotic symptoms induced on tobacco leaves after injection of protein extracts from 1, healthy leaves of *Nicotiana benthamiana* (2 mg/ml); 2, systemically PVX202-infected leaves of *N. benthamiana* (2 mg/ml); 3, systemically PVX202::*hrpZ*$_{Psph}$-infected leaves of *N. benthamiana* (2 mg/ml); 4, purified harpin (100 μg/ml; 5, cell suspension of *Pseudomonas syringae* pv. *phaseolicola* (2×10^8 CFU/ml) in 10 mM $MgCl_2$. Leaf was photographed 24 h after infiltration under white light.

In light of the above results, it was important to establish whether the endogenously produced harpin in PVX-202::*hrpZ*$_{Psph}$-infected plants retained its HR-inducing activity. Total protein extracts were prepared from *N. benthamiana* leaves infected with either PVX202::*hrpZ*$_{Psph}$ or the vector alone. After ammonium sulfate precipitation and extensive dialysis, the extracts were injected into *N. tabacum* cv. Xanthi leaf mesophyll panels. Leaf panels injected with these extracts developed typical HR within 24 h, whereas similar extracts from healthy and PVX202-infected control leaves did not cause necrosis (Fig. 4). Similar results were obtained when the protein extracts were injected into *N. tabacum* cv. W38 and *N. benthamiana* leaves (not shown). These results showed that harpin produced in plants is biologically active. Therefore, the inability of endogenously produced harpin in PVX202::*hrpZ*$_{Psph}$-infected leaves to elicit necrosis is not a result of insufficient quantity or a lack of intrinsic HR elicitor activity.

A most likely explanation of our findings is that the protein does not gain access to the appropriate cellular target(s) (e.g., outside the plant cell). This possibility was tested by infecting *N. benthamiana* plants with the recombinant virus PVX202::*SP-hrpZ*$_{Psph}$ encoding the secretable form of HrpZ. The plants developed mosaic symptoms in upper, noninoculated leaves 8 to 10 days after inoculation and numerous small necrotic lesions 3 to 5 days later. The symptoms occurred predominantly in the basal portion of the leaves (Fig. 5). Neither necrosis nor mosaic developed in the distal to the petiole portion of the leaves, indicating that the ensuing plant response was limiting the spread of the recombinant virus. Immunoblot analysis of total protein extracts from PVX202::*SP-hrpZ*$_{Psph}$-infected plants revealed the presence of immunoreactive bands corresponding in size to SP-HrpZ$_{Psph}$ and HrpZ$_{Psph}$ (not shown), indicating that the protein was indeed produced and the SP extension was properly processed.

It is well documented that in planta expression of bacterial and fungal avirulence genes (e.g., *avrPto*, *avrRpt2*, and *avr9*) leads to necrosis (Hammond-Kosak et al. 1995, Kamoun et al. 1999, Mudgett and Staskawicz 1999, Tobias et al. 1999). Therefore, we compared the severity of necrosis caused by harpin and AvrPto in *N. benthamiana* infected with PVX202::*SP-hrpZ*$_{Psph}$ and PVX202::*avrPto* (Fig. 5). In contrast to the small necrotic lesions observed with PVX202::*SP-hrpZ*$_{Psph}$, PVX202::*avrPto* elicited extensive necrosis over the entire basal portion of the systemically infected leaves 5 to 7 days after inoculation. Furthermore, expression of AvrPto caused death of the entire plant, which was not observed with harpin.

Necrosis caused by the secretable form of harpin is HR specific.

It has been established that *hsr203J* transcripts accumulate specifically in tissues undergoing HR following pathogen infection or harpin injection into leaf intercellular spaces (Pontier et al. 1994). To determine whether the necrosis observed in plants infected with PVX202::*SP-hrpZ*$_{Psph}$ is HR-specific, the induction of gene transcripts was examined by Northern hybridization. Total RNA extracts from *N. benthamiana* leaves systemically infected with PVX202::*hrpZ*$_{Psph}$ and PVX202::*SP-hrpZ*$_{Psph}$ and prepared at various times after inoculation were probed with an *hsr203J* probe (Fig. 6). Similar extracts from plants inoculated 6 h earlier with *P. syringae* pv. *phaseolicola* were also included in the Northern analysis. It proved experimentally difficult, however, to investigate the *hsr203J* transcript accumulation in plants infected with PVX202::*SP-hrpZ*$_{Psph}$ or PVX202::*avrPto* because of the transient induction of this gene and uncertainty about the kinetics of harpin accumulation in the PVX202-infected plants. Nevertheless, *hsr203J* was detected in PVX202::*SP-hrpZ*$_{Psph}$-infected plants but not in those infected with PVX202

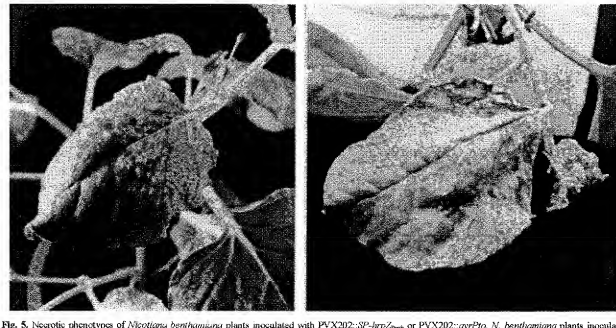

Fig. 5. Necrotic phenotypes of *Nicotiana benthamiana* plants inoculated with PVX202::*SP-hrpZ*$_{Psph}$ or PVX202::*avrPto*. *N. benthamiana* plants inoculated with PVX202::*SP-hrpZ*$_{Psph}$ (left) photographed 12 days after inoculation or with PVX202::*avrPto* (right) and photographed 7 days after inoculation.

(Fig. 6). The *hsr203J* transcript accumulation was observed prior to the onset of necrotic symptoms.

Origin of a truncated form of HrpZPsph produced in planta.

The expression of the *hrpZ*$_{Psph}$ gene in planta generates two forms that differ in apparent molecular mass by approximately 2 kDa. We considered two possible scenarios for the origin of the lower molecular mass species. One possibility is proteolysis, based on the observation that other type III-delivered proteins are cleaved in bacteria (e.g., PopA1, HrpA, DspA, and AvrPphB) (Arlat et al. 1994; Gaudriault et al. 1997; Puri et al. 1996; Roine et al. 1997) or in planta (e.g., AvrRpt2, AvrB, and AvrPphB) (Gopalan et al. 1996; Mudgett and Staskawicz 1999; *our unpublished data*). Another possibility is alternative translation initiation on the basis that an internal ATG codon is present at position 16. Initially we examined whether harpin purified from *E. coli* or synthesized in vitro (TnT system) is cleaved in the presence of leaf protein extract. No cleavage or degradation was observed during 90 min of incubation at 37°C in the presence or absence of leaf protein extract, indicating that neither autoproteolysis nor plant protease-mediated cleavage was involved in the generation of the lower molecular mass form of harpin inside plant cells (Fig. 7A). We next examined whether the lower band may be a product of alternative translation initiation. The ATG codon at position 16 was changed to isoleucine by polymerase chain reaction (PCR) amplification with appropriately designed primers. The resulting construct (pT7-7/*hrpZ-M16I*) was expressed in vitro with the TnT-coupled rabbit reticulocyte lysate system. As expected, full-length HrpZ$_{Psph}$ was produced, whereas the lower band was not detected (Fig. 7B). This indicates that the generation of the lower band seen in the plant is probably a product of internal translation initiation in plant cells.

DISCUSSION

The TTSS of plant-pathogenic bacteria serve as conduits to deliver HR effector proteins to the proper destination in the plant host. Harpins remain the only type III-secreted proteins that are able to elicit HR when administered externally to plant tissues in purified form. The harpins described thus far in different phytopathogenic bacteria share several characteristics (Arlat et al. 1994; Bauer et al. 1995; He et al. 1993; Wei et al. 1992) that are also found in the HrpZ$_{Psph}$ of *P. syringae* pv. *phaseolicola* NPS3121. It is glycine-rich, cysteine-lacking and heat stable. It also lacks a classical signal peptide and is secreted in culture media via the TTSS (*our unpublished data*). Hypotheses have been proposed to correlate the significance of these properties with the biological activity of harpins in the context of plant-bacterium interactions. One of the common characteristics of harpins is the lack of cysteine residues. It has been hypothesized that an unfolded state of harpins may facilitate their movement into the plant cell wall matrix rather than translocate through the Hrp pathway. Several bacterial Avr proteins thought to travel the type III pathway are relatively large and cysteine-rich and appear to interact with intracellular receptors. Direct evidence that harpins interact with extracellular receptors, however, is rather limited.

Our results provide strong evidence that HrpZ$_{Psph}$ is able to elicit necrosis when expressed in planta in sufficient quantity and in a form that can be secreted to the plant cell exterior. We employed two different approaches to express harpin in plant cells: stable transformation and transient expression through PVX infection and agroinfiltration. Stable transformants expressing HrpZ$_{Psph}$ or SP-HrpZ$_{Psph}$ did not show any necrotic spots after tetracycline treatment in hydroponic culture, yet HrpZ$_{Psph}$ protein was detected in leaves of transformants at levels 10-fold higher than those produced by bacteria in situ in standard HR tests with limit dilution of the inoculum. Under these conditions, cardinal molecular events associated with HR such as induction of *hsr203J* transcripts were not detected. Furthermore, agrobacteria expressing either the secretable or canonical form of harpin did not elicit HR following agroinfiltration in tobacco plants.

Assuming that the expression level of HrpZ$_{Psph}$ in stably transformed and agroinfiltrated leaves was not sufficient, we employed another approach based on potato virus X to in-

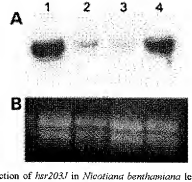

Fig. 6. Induction of *hsr203J* in *Nicotiana benthamiana* leaves infected with PVX202::*SP-hrpZ*$_{Psph}$. Lane 1, Total RNA from leaves infected with *Pseudomonas syringae pv. phaseolicola* 6 h after injection. Lane 2, Healthy leaves. Lane 3, Systemically infected leaves with PVX 12 days after inoculation. Lane 4, Systemically infected leaves with PVX-202::*SP-hrpZ*$_{Psph}$ and analyzed by Northern blot analysis. Each lane contained 30 µg of total RNA. **A,** Blot was hybridized with an *hsr203J* cDNA probe. **B,** Equal loading was verified before blotting by visualizing rRNA in a gel stained with ethidium bromide.

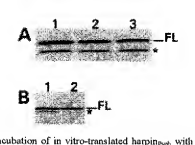

Fig. 7. A, Incubation of in vitro-translated harpin$_{Psph}$ with and without total protein extracts from leaves of *Nicotiana benthamiana*. Lane 1, Input HrpZ$_{Psph}$. Lanes 2 and 3, HrpZ$_{Psph}$ incubated for 90 min at 37°C with and without plant extracts, respectively. **B,** In vitro expression of wild-type HrpZ$_{Psph}$ and M16I mutant derivative. Aliquots (5 µl) of the reactions were taken at 90 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. Lane 1, input HrpZ$_{Psph}$. Lane 2, M16I mutant. FL = full length HrpZ$_{Psph}$, and * = product of internal initiation. Translations of wild-type and mutant HrpZ$_{Psph}$ were performed in the TnT rabbit reticulocyte lysate system in the presence of [^{35}S]-methionine with 1 µg each of pT7-7/*hrpZ* and pT7-7/*hrpZ*-M16I plasmids as templates.

crease the amount of harpin produced in the plant. The canonical form of $HrpZ_{Psph}$ failed to elicit HR, even though the protein was biologically active and accumulated at levels that were judged to be sufficient on the basis of the amount of pure harpin able to induce HR in the standard leaf-injection assay. These results clearly show that $HrpZ_{Psph}$ does not act inside the plant cells to signal HR. We can exclude the possibility that the lower molecular weight form of harpin detected in planta may interfere with full elicitor activity of the canonical protein. Published evidence (Alfano et al. 1996) shows that elicitor activity resides in multiple regions of $HrpZ_{Pss}$. Furthermore, our own data (*unpublished*) shows that removal of the first 150 residues from $HrpZ_{Psph}$ did not alter its elicitor activity in tobacco leaf-injection assays.

In contrast to the results of the canonical form of harpin, necrosis was observed when the secretable form of the protein was expressed via the PVX vector. The induction of *hsr203J* confirmed that the necrosis was HR-specific. Additionally, detection of the transcript was not reproducible in our experiments. This could be explained by a combination of factors, including the asynchronous nature of the HR response elicited by PVX infection, the inability to predict the onset of transgene expression, and the temporal nature of *hsr203J* expression (Pontier et al. 1994). It is worth noting that the induction of this gene occurs prior to the onset of tissue necrosis. Our results show that overexpression of $HrpZ_{Psph}$ causes necrosis only when the protein is produced in sufficient quantity and secreted by the plant cells. This finding suggests that the putative receptor of $HrpZ_{Psph}$ is localized extracellularly, either in the plant cell wall or on the plant cell membrane. The existence of an extracellular receptor also has been suggested by the study of Hoyos et al. (1996), which proposed that $harpin_{Pss}$ may interact with a plant cell wall component(s) but not with the plasma membrane. The possibility that harpin may bind to the plasma membrane could not be discounted as a result of the technical limitations of the approach. For example, destruction of or conformational changes in harpin-binding sites may occur during enzymatic protoplast isolation or competition of cell wall peptides may be released by the enzymatic treatment with the harpin-binding sites. Our experimental approach yields results that are closer to the in vivo state because the integrity and functional relations between plant cell wall and plasmalemma are not disturbed. Our findings are further corroborated by a recent study (Lee et al., *unpublished data*), which showed that $HrpZ_{Psph}$ is stably integrated into artificial membranes and has pore-forming activity.

The necrosis caused by harpin expression showed differences compared with that caused by the expression of *avrPto*. The necrotic symptoms caused by harpin were milder and slower to appear compared with those caused by AvrPto. Several possibilities can be envisioned, such as that the secreted form of $HrpZ_{Psph}$ is expected after secretion to yield a polypeptide with an N terminus slightly different from that of the canonical harpin (substitution of four N-terminal amino acids of harpin with six different residues). The absence of the four canonical residues is unlikely to affect HR-elicitor activity for reasons mentioned earlier. The possibility that the six new residues might render the protein intrinsically less active, however, cannot be totally discounted. Note that a significant portion of SP-$HrpZ_{Psph}$ remains intracellular and therefore unavailable for interaction with external binding sites. Whether the intracellular harpin can interfere with HR elicitation is an interesting but open question. Finally, it has been reported that $HrpZ_{Pss}$ and $HrpN_{Ea}$ induce systemic acquired resistance (SAR) in cucumber and arabidopsis, respectively (Dong et al. 1999; Strobel et al. 1996). It is possible that the mild nature of the necrotic symptoms seen in our work may be the result of an early induction of SAR by endogenously produced $HrpZ_{Psph}$ that interferes with HR development.

The main conclusions from this study are that $HrpZ_{Psph}$ expressed endogenously in *N. benthamiana* causes HR only when it is produced in a secretable form via the PVX vector and in a sufficient quantity. The protein evidently interacts with an externally exposed receptor(s), which may be located in the plasmalemma or the cell wall. The nature of this receptor(s) and the molecular mechanisms underlying harpin perception by the plant cell remain to be elucidated.

MATERIALS AND METHODS

Plant material and bacterial strains.

N. tabacum cv. W38 $TetR^+$ (Gatz et al. 1991), *N. tabacum* cv. Xanthi, and *N. benthamiana* were grown under controlled greenhouse conditions. Infiltration of tobacco leaves with bacteria, purified harpin, or plant protein extracts was carried out as described (He et al. 1993). *E. coli* strains and *A. tumefaciens* C58C1 carrying the disarmed Ti plasmid pGV2260 (Deblaere et al. 1985) were routinely grown on Luria-Bertani (LB) agar or broth at 37°C (Sambrook et al. 1989). *P. syringae* pv. *phaseolicola* (NPS3121) was grown in King's B broth at 30°C (King et al. 1954). For in vitro expression of *P. syringae* pv. *phaseolicola hrp* genes, cultures were grown in *hrp*-inducing fructose minimal medium of Huynh et al. (1989) at 30°C. Antibiotics were used at the following concentrations per milliliter: 100 µg of ampicillin, 100 µg of kanamycin, 100 µg of carbenicillin, 50 µg of rifampicin, and 50 µg of hygromycin.

Plasmid constructions.

The plasmid constructs were made according to standard molecular biological procedures as described by Sambrook et al. (1989).

pUC-A7-TX/SP. The coding region of the tobacco pathogenesis-related protein PR1a signal peptide portion (Pfitzner et al. 1987) was isolated by PCR amplification from *N. tabacum* cv. Xanthi genomic DNA. The primers (5′-GCCGCG-GGTACCAAGCTTTCCTATAGTCATGGG-3′ and 5′-CTCT-GAGGGATCCTTGTTGAGAGTTTTGGGCACG-3′) were designed to contain restriction sites (*KpnI* and *BamHI*, underlined) to facilitate cloning in the corresponding sites of the pUC-A7-TX vector (Roder et al. 1994). A 50-µl PCR of reaction mixture containing 10 pmol of each primer, 1 µg of *N. tabacum* genomic DNA; 10 mM KCl, 20 mM Tris-HCl, pH 8.8; 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 0.1% Triton X-100; 200 µM dNTPs; and 2 units of Deep Vent DNA polymerase (New England Biolabs Inc., Beverly, MA, U.S.A.) were subjected to 30 cycles of amplification (1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C). The PCR product was digested with *KpnI* and *BamHI* and cloned into *KpnI/BamHI*-digested pUC-A7-TX.

pUC-A7-TX/*hrpZ*. The coding region of the *hrpZ*$_{Psph}$ gene from *P. syringae* pv. *phaseolicola* NPS3121 (343 codons) was

obtained from the pT7-7/*hrpZ* construct (Tampakaki et al. 1997) after digestion with *Nde*I and *Eco*RV. The protruding ends were filled in with Klenow DNA polymerase, and the digested fragment was cloned into the *Sma*I-digested pUC-A7-TX vector. The sequence of the $hrpZ_{Psph}$ gene will be published separately.

pUC-A7-TX/*SP-hrpZ*. The construct pUC-A7-TX/SP was digested with *Bam*HI, treated with mung bean nuclease to produce blunt ends, and digested with *Pst*I. The coding region of the $hrpZ_{Psph}$ gene was obtained from pUC-A7-TX/*hrpZ* after digestion with *Dde*I (Klenow fill-in) and *Pst*I. This fragment, which encoded the $HrpZ_{Psph}$ downstream from the fifth amino acid residue, was ligated to the above vector fragment. The resulting plasmid construct, pUC-A7-TX/*SP-hrpZ*, encodes a translational fusion between the PR1a signal sequence and the $hrpZ_{Psph}$ gene. The fused protein consists of 375 residues. The peptide expected after removal of the signal peptide (345 residues) includes six amino acids (QNSQQV), which substitute for the first four residues of harpin (MQSL).

pBIN-Hyg-TX/*hrpZ*. The $hrpZ_{Psph}$ gene, together with the modified CaMV35S promoter and the *ocs* transcriptional terminator, were excised from pUC-A7-TX/*hrpZ* as an *Eco*R–*Hin*dIII fragment and inserted into the corresponding sites of the binary vector pBin-Hyg-TX (Gatz, *personal communication*), conferring hygromycin resistance to transgenic plants.

pBIN-Hyg-TX/*SP-hrpZ*. As above, the chimeric gene *SP-hrpZ* was excised from pUC-A7-TX/*SP-hrpZ* as an *Eco*RI–*Hin*dIII fragment and inserted into the binary vector pBIN-Hyg-TX, which was linearized with *Eco*RI/*Hin*dIII.

PVX202::*hrpZ*. The fragment *Hpa*I/*Hin*dIII (Klenow fill-in) from construct pUC-A7-TX/*hrpZ* was cloned into the *Nru*I site of the viral expression vector PVX202 (Sablowski et al. 1995).

PVX202::*SP-hrpZ*. The fragment *Hin*dIII (Klenow fill-in) from the construct pUC-A7-TX/*SP-hrpZ* was cloned into the *Nru*I site of the viral expression vector PVX202.

PVX202::*avrPto*. The coding region of the *avrPto* gene was isolated by PCR amplification from the pDSK519/*avrPto* plasmid. The primers 5′-TGTACTCGCGAGGGTATACGAA-TGGG-3′ and 5′-GCCTCGCGAGTCGACATTATGACGCC-3′ were designed to contain the restriction site *Nru*I (underlined) to facilitate cloning in the *Sma*I of pUC18 plasmid. The *avrPto* was excised from pUC18/*avrPto* as an *Nru*I fragment and inserted into the *Nru*I site of PVX202 vector.

pUC18/*hsr203J*. A portion of the coding region (nucleotides 1443 to 2041) of *hsr203J* corresponding to the published sequence (accession X77136) was isolated by PCR amplification from the genomic DNA of *N. tabacum* cv. Xanthi using primers 5′-CGCGGATCCGGCTGGCTTAGAG-TTTTC-3′ and 5′-TCCGGGATCCTCCGATAGGACCGCA-CG-3′. Both primers were designed to contain the *Bam*HI restriction site (underlined) to facilitate cloning into the corresponding site of the pUC18 plasmid.

pT7-7/*hrpZ*-M16I. The plasmid pT7-7/*hrpZ* was used to mutagenize the methionine codon at position 16 (based on the $hrpZ_{Psph}$ sequence, *our unpublished data*) to an isoleucine codon in accordance with the protocol described by Fisher and Pei (1997). The mutagenesis was performed by PCR amplification with the primers 5′-CG*ATC*GCGCTCGTTCTGAT-CCGTCC-3′ (forward, mutagenic primer) and 5′-ACGGGCT-TTCGAGCGTGCTGCTG-3′ (divergent, nonoverlapping primer). The mutagenic primer contains the mutant codon for isoleucine (italics) and a *Pvu*I restriction site (underlined). The mutation was confirmed b *Pvu*I digestion and sequencing.

Plant transformation.

Healthy, well-expanded leaves from 6-week-old plants of *N. tabacum* cv. W38 TetR[+] were used for *Agrobacterium* transformation by leaf disk method as described by Horsch et al. (1988). The binary plasmid constructs were introduced into *Agrobacterium tumefaciens* by triparental conjugation (Rogers et al. 1988). Colonies were selected on LB medium with 1.5% Bacto agar, 50 μg of rifampicin per ml, 100 μg of carbenicillin per ml, and 100 μg of kanamycin per ml. The transconjugants were used to transform *N. tabacum* cv. W38 TetR[+] plants. Hygromycin-resistant transformants were grown in vitro on Murashige–Skoog media (Murashige and Skoog 1962) with appropriate hormone regimes for shoot and root formation and subsequently maintained in hydroponic culture under controlled conditions (25°C, 16-h photoperiod) or in pots in the greenhouse.

Protein extraction and analysis.

Leaves were immediately frozen in liquid nitrogen after collection and broken with a mortar and pestle. Total soluble protein was extracted in a buffer containing 200 mM Tris-HCl, pH 8; 1 mM EDTA; 5 mM DTT; 0.5 mM PMSF; 10 μM leupeptin; 10% glycerol; and 0.25% Triton X-100. Samples were incubated with shaking on ice for 30 min and centrifuged (14,000 rpm, 15 min, 4°C). The supernatants were then collected and kept at −20°C in small aliquots.

Immunoblot analysis.

Accumulation of the $hrpZ_{Psph}$ gene product was assayed in hygromycin-resistant, regenerated plantlets by quantitative immunoblotting. Soluble leaf protein was extracted, separated on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels, and transferred to the nitrocellulose membrane by standard procedures. The membranes were blocked and incubated with an anti-$HrpZ_{Psph}$ antibody (1:20,000) and developed with an alkaline phosphatase-conjugated antibody with nitroblue tetrazolium and 5-bromo-4-chloro-3-iodyl phosphate, in accordance with the supplier's instructions. To detect PVX coat protein accumulation, the membranes were incubated with an anti-PVX antibody (1:2,000 dilution) and developed as above.

Plant nucleic acid extraction and analysis.

Plant DNA was isolated with the method described by Rogers and Bendich (1988). Total RNA was isolated from tobacco leaf tissues with Tri Reagent (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.). Leaves were frozen in liquid nitrogen immediately after harvesting and kept at −80°C for further extraction. Thirty micrograms of total RNA from each sample was loaded on a denaturing 1.2% formaldehyde-agarose gel, separated by electrophoresis, and transferred onto a nylon filter (Hybond-N, Amersham, Arlington Heights, IL, U.S.A.). Following UV cross-linking, the filters were prehybridized for 2 h at 65°C in 0.5 M phosphate buffer, pH 7.2, containing 7% SDS, 1% bovine serum albumen, and 1 mM EDTA (Church and Gilbert 1984). The filters were hybridized in the same buffer with cDNA and labeled with ^{32}P-dATP and ^{32}P-dCTP by random priming procedure (Sambrook et al. 1989). The

membranes were washed in 0.4 M phosphate buffer, pH 7.2, containing 5% SDS and 1 mM EDTA at 65°C.

Tetracycline induction.

Transgene induction in *N. tabacum* cv. W38 TetR$^+$ primary transformants (T_0) or T_1 progeny was accomplished by supplying tetracycline at a concentration of 1 mg per liter, either by vacuum infiltration of detached leaves or in hydroponic culture in Hoagland buffer as described by Gatz et al. (1991).

PVX-inoculations.

Four-week-old *N. benthamiana* plants were inoculated with the viral expression vector PVX202, PVX202::*hrpZ*$_{Psph}$, or PVX202::*SP-hrpZ*$_{Psph}$ by gently rubbing leaves dusted with Carborundum, as described by Sablowski et al. (1995). Two leaves of each plant were inoculated with 5 to 10 µg of Qiagen-purified (Chatsworth, CA, U.S.A.) DNA dissolved in 30 µl of 50 mM sodium phosphate buffer, pH 7. Two weeks later, young, systemically infected leaves were harvested and immediately homogenized in the same buffer or stored at −80°C for protein and RNA extraction and plant inoculations.

In vitro transcription and translation.

Wild-type HrpZ and the mutant M16I were expressed from the pT7-7 templates by the TnT T7 rabbit reticulocyte lysate-coupled transcription–translation system (Promega, Madison, WI, U.S.A.) in the presence of [^{35}S] methionine (final concentration, 1 mCi/ml) (Amersham), in accordance with the manufacturer's instructions. Reactions were incubated at 30°C for 90 min. Aliquots of the reactions (5 µl) were run on 12% SDS-polyacrylamide gels, which were dried and analyzed with autoradiography.

ACKNOWLEDGMENTS

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Exhibit 7 - Declaration of Zhong-Min Wei Under 37 C.F.R. § 1.132 (Aug. 11, 2004)

PATENT
Docket No.: **19603/2986 (CRF D-1940B)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Qiu et al.	Examiner: A. Kubelik
Serial No.	:	09/766,348	Art Unit: 1638
Cnfrm. No.	:	7683	
Filed	:	January 19, 2001	
For	:	HYPERSENSITIVE RESPONSE INDUCED RESISTANCE IN PLANTS BY SEED TREATMENT	

DECLARATION OF ZHONG-MIN WEI UNDER 37 C.F.R. § 1.132

I, ZHONG-MIN WEI, pursuant of 37 C.F.R. § 1.132, declare:

1. I received a B.S. degree in Biology from Zhejiang University, Zhejiang, China in 1982, an M.S. degree in Plant Pathology from Nanjing Agricultural University, Nanjing, China in 1984, and a Ph.D. degree in Molecular Biology from Nanjing Agricultural University and Academy of Science, Shanghai, China in 1987.

2. I am currently employed as Chief Scientific Officer and Vice President of Research and Development at EDEN Bioscience Corporation in Bothell, Washington.

3. I am an inventor of the above-identified application.

4. I am presenting this declaration to show that hypersensitive response elicitors from a diverse range of plant pathogenic bacteria (1) are an art-recognized class of proteins where results achieved with one such protein would be expected when other proteins in this class are used and (2) share the unique ability to cause distinct plant responses. Specifically, treatment of a variety of plants and plant seeds with hypersensitive response elicitors was shown to induce plant disease resistance as compared with plants and plant seeds not treated with a hypersensitive response elicitor; and transgenic expression of hypersensitive response elicitors in transgenic plants was shown to induce plant disease resistance as compared to null transfected plants or wild-type plants.

5. In plants, the hypersensitive response phenomenon results from an *incompatible* interaction between plant pathogens and non-host plants. As explained in Gopalan et al., "Bacterial Genes Involved in the Elicitation of Hypersensitive Response and Pathogenesis," *Plant Disease* 80: 604-10 (1996) ("Gopalan") (attached hereto as **Exhibit 1**), these types of interactions involve, for example, a bacterial plant pathogen attempting to infect a host plant, and the host plant preventing proliferation of the pathogen by the collapse and death, or necrosis, of plant leaf cells at the site of infection. This is distinct from a *compatible* interaction between a bacterial plant pathogen and a host plant in which the bacteria is capable of proliferation, resulting in the spread of the pathogen throughout the plant and the manifestation of disease symptoms. *Id.* at 604.

6. Hypersensitive response elicitors within a given genus are often homologous to elicitors from different pathogenic species and strains of the same genus. For example, homologs of hypersensitive response elicitors from *Erwinia amylovora* and *Pseudomonas syringae* have been identified in different bacteria species and strains from the genera *Erwinia* and *Pseudomonas*, respectively. *See* Gopalan.

7. In addition, numerous reported studies confirm that a gene encoding a hypersensitive response elicitor from a particular source genus can be used to isolate a corresponding hypersensitive response elicitor gene from different species and strains of that same genus. For example, in Bauer et al., "*Erwinia chrysanthemi* $Harpin_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis," *MPMI* 8(4): 484-91 (1995) ("Bauer") (attached hereto as **Exhibit 2**), the *Erwinia amylovora* hypersensitive response elicitor encoding gene was used as a probe to isolate, clone, and sequence the gene encoding the *Erwinia chrysanthemi* hypersensitive response elicitor, as follows:

> The cosmids were probed in colony blots with a 1.3-kb *Hind*III fragment from pCPP1084, which contains the *E. amylovora* *hrpN* gene (Wei et al. [, "Harpin Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (]1992[)]). pCPP2157, one of the three cosmids hybridizing with the probe, was digested with several restriction enzymes, and the location of the $hrpN_{Ech}$ gene in those fragments was determined by probing a Southern blot with *E. amylovora* *Hind*III fragment. Two fragments, each containing the entire $hrpN_{Ech}$ gene, were subcloned into different vectors: pCPP2142 contained an 8.3-kb *Sal*I fragment in pUC119 (Vieira and Messing [,"Production of Single-Stranded Plasmid DNA," Methods Enzymol., 153:3-11(] 1987[)]), and pCPP2141 contained a 3.1-kb *Pst*I fragment in pBluescript II SK(-) (Stratagene, La Jolla, CA).

Sequence of $hrpN_{Ech}$

The nucleotide sequence of a 2.4-kb region of pCPP2141 encompassing $hrpN_{Ech}$ was determined. The portion of that sequence extending from the putative ribosome-binding site through the $hrpN_{Ech}$ coding sequence to a putative rho-independent terminator is presented in Figure 1.

See page 485.

8. In the same manner as described in Bauer *supra*, Cui et al., "The RsmA$^-$ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress $hrpN_{Ecc}$ and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," *MPMI* 9(7): 565-73 (1996) ("Cui") (attached hereto as **Exhibit 3**) demonstrates that the gene encoding the *Erwinia carotovora* hypersensitive response elicitor can be isolated, sequenced, and cloned by using the *Erwinia chrysanthemi* hypersensitive response elicitor encoding gene to probe the genomic library of *Erwinia carotovora*. Further, Cui (at page 572) states the following:

> The genomic library of *E. carotovora* subsp. *carotovora* strain Ecc71 in pLARF5 was screened by in situ colony hybridization with a 0.75-kb internal *Cla*I fragment of *hrpN* of *E. chrysanthemi* (Bauer et al.[, "*Erwinia chrysanthemi* $Harpin_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (]1995). Two cosmids, pAKC921 and pAKC922, that hybridized with the probe were isolated. The subclones (pAKC923 and pAKC924, Table 1) carrying *hrpN* DNA were used for sequence analysis.

9. The gene encoding the hypersensitive response elicitor of *Erwinia amylovora* has also been used as a probe to isolate and clone the gene encoding the hypersensitive response elicitor of *Erwinia stewartii*. It was found that antibodies raised against the hypersensitive response elicitor of *Erwinia stewartii* cross-reacted with the hypersensitive response elicitor of *Erwinia amylovora*. *See* Ahmad et al., "Harpin Is Not Necessary for the Pathogenicity of Maize," *8th Int'l Cong. Molec. Plant Microbe Inter.* July 14-19, 1996 ("Ahmad") (attached hereto as **Exhibit 4**).

10. The genes encoding the HrpN hypersensitive response elicitor from several strains of *Erwinia pyrifolia* have since been cloned. As reported in Jock et al., "Molecular Differentiation of *Erwinia amylovora* Strains from North America and of Two Asian Pear Pathogens by Analyses of PFGE Patterns and *hrpN* genes," *Environ. Microbiol.* 6(5): 480-490 (2004) ("Jock") (attached hereto as **Exhibit 5**), the *hrpN* genes were amplified with PCR consensus primers that were deduced by comparison of the known nucleotide

sequences of *Erwinia amylovora hrpN* and *Erwinia stewartii hrpN*. Indeed, Jock (at page 489) recites the following:

> *Erwinia pyrifoliae* and the *Erwinia* strains from Japan were considered to be sufficiently related to *E. amylovora* to amplify their genes with the *Erwinia* PCR consensus primers given above. This was indeed possible and allowed cloning and sequencing of their *hrpN* DNA fragments. . . .

11. Similar findings were reported for hypersensitive response elicitors from the genus *Pseudomonas*. An internal fragment of the hypersensitive response elicitor from *Pseudomonas syringae* pv. *syringae* (i.e., *hrpZ*) was used to identify and isolate the hypersensitive response elicitors from *P. syringae* pv. *glycinea* and *P. syringae* pv. *tomato*. Significant amino acid sequence similarities were identified between the various *Pseudomonas syringae* elicitors. *See* Preston et al., "The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae, glycinea,* and *tomato* Are Encoded by an Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive Response in Tomato But Not Soybean," *MPMI* 8(5): 717-32 (1995) ("Preston") (attached hereto as **Exhibit 6**).

12. The genes encoding hypersensitive response elicitors are positioned within the *hrp* gene cluster or proximate to the *hrp* gene cluster in *hrp* regulons. For example, *hrpN* from *Erwinia amylovora* was located within the *hrp* gene cluster, as was *hrpZ* from *Pseudomonas syringae*. The *popA* gene, encoding a hypersensitive response elicitor from *Pseudomonas solanacearum*, was located on the left flank of the *hrp* gene cluster within a *hrp* regulon. *See* Bonas, "*hrp* Genes of Phytopathogneic Bacteria," *Current Topics in Microbiology and Immunology* 192: 79-98 (1994) ("Bonas I") (attached hereto as **Exhibit 7**) and Alfano et al., "The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death," *Journal of Bacteriology* 179: 5655-5662 (1997) ("Alfano") (attached hereto as **Exhibit 8**). Similar to the *popA* gene, *hreX*, the gene encoding the hypersensitive response elicitor from *Xanthomonas campestris*, was located on the left flank of the *hrp* gene cluster. *See* Swanson et al., "Isolation of the *hreX* Gene Encoding the HR Elicitor Harpin (Xcp) from *Xanthomonas campestris* pv. *pelargonii*," *Phytopathology* 90: s75 (1999) ("Swanson") (attached hereto as **Exhibit 9**).

13. The characteristics that distinguish hypersensitive response elicitors as a distinct class of molecules are clearly apparent when considering the different elicitors' secretion mechanisms, regulation, biochemical characteristics, and biological activities.

14. Substantially all hypersensitive response elicitors identified have been shown to be secreted through the type III, *hrp* dependent secretion pathway. The type III secretion pathway is a highly conserved and unique mechanism for the delivery of pathogenicity related molecules in gram-negative bacteria. The *hrp* gene cluster is largely composed of components of the type III secretion system. *See* Bogdanove et al., "Unified Nomenclature for Broadly Conserved *hrp* Genes of Phytopathogenic Bacteria," *Molec. Microbiol.* 20:681-83 (1996) (attached hereto as **Exhibit 10**); and Alfano.

15. Regulation of the genes encoding the *hrp* gene cluster, and subsequently the genes encoding the components of the type III secretion system and hypersensitive response elicitors, is controlled by environmental factors. Specifically, transcriptional expression of these genes is induced under conditions that mimic the plant apoplast, such as low concentrations of carbon and nitrogen, low temperature, and low pH. *See* Wei et al., "Regulation of hrp Genes and Type III Protein Secretion in *Erwinia amylovora* by HrpX/HrpY, a Novel Two-Component System, and HrpS," *MPMI* 13(11): 1251-1262 (2000) ("Wei I") (attached hereto as **Exhibit 11**); and Bonas I.

16. Biochemically, hypersensitive response elicitors have a number of common characteristics. These include being glycine rich, heat stable, hydrophilic, lacking of an N-terminal signal sequence, and susceptible to proteolysis. *See* Bonas, "Bacterial Home Goal by Harpins," *Trends Microbiol* 2: 1-2 (1994) (attached hereto as **Exhibit 12**); Bonas I; Gopalan; and Alfano.

17. In addition, hypersensitive response elicitors share a unique secondary structure that has been associated with these elicitors' distinct biological activities (described below). The structure has two primary components, an alpha helix unit and a relaxed acidic unit having a sheet or random turn structure. In the absence of one or both of these components, hypersensitive response elicitation does not occur. *See* WO 01/98501 to Fan et al. (attached hereto as **Exhibit 13**).

18. In addition to eliciting the hypersensitive response in a broad range of plant species, as explained by Wei et al., "Harpin from *Erwinia amylovora* Induced Plant Resistance," *Acta Horticulture* 411: 223-225 (1996) ("Wei II") (attached hereto as **Exhibit 14**) and by Alfano, hypersensitive response elicitors also share the ability to induce specific plant responses. The induction of plant disease resistance, plant growth enhancement, and plant stress resistance are three plant responses that result from treatment of plants or plant seeds with a hypersensitive response elicitor from a gram-negative plant pathogen.

19. As described in Wei II, treatment of plants with the hypersensitive response elicitor HrpN from *Erwinia amylovora* resulted in disease resistance to a broad range of plant pathogens. For example, HrpN induced disease resistance to southern bacterial wilt (*Pseudomonas solanacearum*) in tomato, tobacco mosaic virus in tobacco, and bacterial leaf spot (*Gliocladium cucurbitae*) in cucumber.

20. The hypersensitive response elicitor HrpZ from *Pseudomonas syringae* was reported to induce disease resistance in cucumber to a diverse range of pathogens, including the fungal disease *Colletotrichum lagenarium*, tobacco necrosis virus, and bacterial angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*). *See* Strobel et al., "Induction of Systemic Acquired Resistance in Cucumber by *Pseudomonas syringae* pv. *syringae* 61 $HrpZ_{Pss}$ Protein," *Plant Journal* 9(4): 431-439 (1996) (attached hereto as **Exhibit 15**).

21. The hypersensitive response elicitor HrpZ from *Pseudomonas syringae* was reported to induce disease resistance in transgenic tobacco to powdery mildew (*Erysiphe cichoracearum*), and in transgenic rice to rice blast fungus (*Magneporthe grisea*). *See* U.S. Patent Application Publ. No. 2004/0073970 to Takakura et al. (attached hereto as **Exhibit 16**) at Example 4. The HrpZ-expressing transgenes included transgenes with either a weak or a strong constitution promoter, an inducible promoter, or a tissue-specific promoter. *Id.* at Example 3.

Hypersensitive Response Elicitors Induce Plant Disease Resistance

22. As demonstrated by the following experimental evidence in paragraphs 23 and 24 below, treatment of tomato and tobacco plants with the hypersensitive response elicitor HreX from *Xanthomonas campestris* pv. *pelargonii* induced disease resistance in the plants against bacterial wilt and tobacco mosaic virus.

23. The induction of disease resistance in tomato against bacterial wilt (caused by the pathogenic bacterium *Pseudomonas solanacearum* K_{60}) was investigated as follows. Approximately 30 days after sowing, tomato plants were sprayed with either a dilution of HreX or 5 mM potassium phosphate buffer, pH 6.8 (the same buffer used to dilute the HreX solution). Six days after treatment, inoculation was performed by slicing the soil of the pot containing the tomato plant 4 times and applying 40 ml of solution containing 1×10^6 colony forming units ("cfu") per ml of *P. solanacearum* K_{60} to the soil. Disease severity ratings were recorded at 7, 9, and 13 days after inoculation ("DAI"), as shown below in

Table 1. As these results demonstrate, plants treated with HreX exhibited substantially more disease resistance than the buffer-treated control plants.

Table 1. ***Pseudomonas solanacearum*** **Disease Resistance from Treatment of Tomato with HreX.**

Treatment Groups[a]	Disease Index (7 DAI)	Disease Index (9 DAI)	Disease Index (12 DAI)	% Difference (12 DAI)
HreX	0.12	0.22	0.22	38.89
Buffer	0.16	0.3	0.36	na

[a]Each group consisted of 1 pot containing 10 plants.

24. Experiments examining the induction of systemic disease resistance in tobacco from treatment with HreX were conducted as follows: Diluted HreX was sprayed on all but the bottom most full-sized leaf of six- to eight-week-old tobacco plants (Xanthi). The bottom most full-sized leaf was covered during spraying so as not to receive residual spray. Three days after the spray treatment, the unsprayed leaf and the leaf opposite it, were lightly dusted with diatomaceous earth. Thereafter, 20 µl of a 1.7 µg/ml solution of tobacco mosaic virus ("TMV") was applied to both leaves dusted with diatomaceous earth. The TMV was gently and evenly spread across the leaves. Approximately 5 minutes after inoculation, the plants were lightly rinsed to remove the diatomaceous earth. Three days after inoculation, the number of TMV lesions on the unsprayed and sprayed leaves for each plant was recorded, as shown below in Table 2. As these results demonstrate, plants treated with HreX exhibited substantially more disease resistance than the buffer-treated control plants.

Table 2. Tobacco Mosaic Virus Resistance in Tobacco from Treatment with HreX.

Treatment Groups	Number of TMV Lesions on Leaf									
	Treated leaves					Untreated leaves				
	Plant No. 1	Plant No. 2	Plant No. 3	Avg. No.	% Difference	Plant No. 1	Plant No. 2	Plant No. 3	Avg. No.	% Difference
HreX	5	7	8	6.67a	93.37	41	22	20	27.67a	76.49
Buffer Control	107	99	96	100.67b	na	124	106	123	117.67b	na

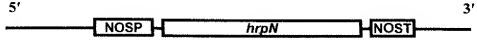
Transformation of Plants and Plant Seeds with a DNA Molecule Encoding a Hypersensitive Response Elicitor Protein

25. In order to investigate whether transforming a plant or plant seed with a DNA molecule encoding a hypersensitive response elicitors results in specific plant

responses, several transformation constructs containing the *hrpN* gene from *Erwinia amylovora* were generated, as described in paragraphs 26-27 below.

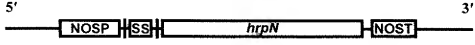
26. A first *hrpN* transformation construct was assembled to include the open reading frame from of the *hrpN* gene inserted behind a nopaline synthase (NOS) promoter, designated NOSP in Figure 1 below, and immediately in front of a NOS terminator, designated NOST in Figure 1 below. The NOS promoter is considered a weak constitutive promoter and has been previously identified. *See* Koncz et al., "The Opine Synthase Genes Carried by Ti Plasmids Contain All Signals Necessary for Expression in Plants," *EMBO J.* 2(9):1597-1603 (1983) (attached hereto as **Exhibit 17**).

Figure 1. Schematic of NOSP-*hrpN*-NOST Transformation Construct.



27. A second *hrpN* transformation construct was assembled that differed from the construct described in paragraph 25 by the insertion of a tobacco *pr1b* signal sequence, designated SS in Figure 2, between the NOS promoter and *hrpN* open reading frame. The *pr1b* signal sequence has been previously identified. *See* Lund & Dunsmuir, "A Plant Signal Sequence Enhances the Secretion of Bacterial ChiA in Transgenic Tobacco," *Plant Mol. Biol.* 18:47-53 (1992) (attached hereto as **Exhibit 18**).

Figure 2. Schematic of NOSP-SS-*hrpN*-NOST Transformation Construct.

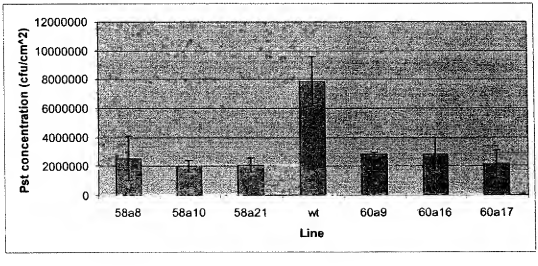


Experimental Evidence Showing Disease Resistance In *hrpN* Transgenic Plants

28. As demonstrated by the following experimental evidence in paragraphs 29-30 below, plants grown from seeds harvested from plants transformed with a DNA molecule encoding the hypersensitive response elicitor HrpN from *Erwinia amylovora* exhibited enhanced disease resistance.

29. *Arabidopsis* Col-0 was transformed with the transformation constructs described above. The constructs were transformed with standard procedures utilizing *Agrobacterium* transfection. Plants designated 58a8, 58a10, and 58a21 were transformed with the construct described in paragraph 26. Plants designated 60a9, 60a16, and 60a17 were transformed with the construct described in paragraph 27. High *hrpN* expression transgenic lines were selected by Northern analysis. The lines were confirmed to be homozygous by selection on kanamycin. Prior to initiation of the growth assays, the seeds of each transgenic line and the wild type *Arabidopsis* were sterilized and subjected to a vernalization treatment in which the seeds were placed at 4°C for approximately four days. All plants were maintained in identical conditions: 16 hours daylight period, 23°C (day)/ 20°C (night), and 50% humidity. Approximately four week after sowing, plants were infiltrated with 10^6 cfu/ml of *Pseudomonas syringae* pv. *tomato* DC3000. Four to six days after inoculation, bacterial concentration were calculated by harvesting 1 cm^2 of infected leaf tissue, macerating the tissue in 10 mM MgCl, and dilution plating the cell/leaf suspension on King's B plates. Bacterial concentrations in wild type and transgenic lines are shown in Figure 3 below. The data in Figure 3 represents the average of three plants per line and six leaves per plant. Disease proliferation was approximately 70% lower in *hrpN* transgenic plant compared to non-transgenic wild type plants.

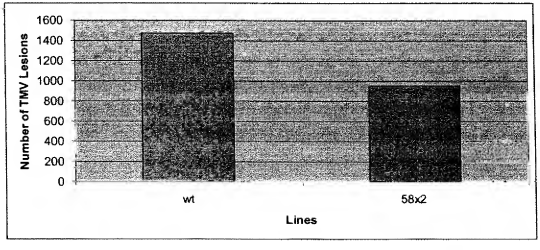
Figure 3. Disease Resistance in *hrpN* Transgenic vs. Wild Type *Arabidopsis*



30. Tobacco (Xanthi NN) was transformed with the transformation constructs described above. The constructs were transformed with standard procedures

utilizing *Agrobacterium* transfection. Plants designated 58x2 were transformed with the construct described in paragraph 27. All seeds and plants were maintained in identical conditions: 12 hours daylight period, 26 °C (day)/ 28°C (night), and 50% humidity. Plants were inoculated with TMV as follows. Four leaves per plant were lightly dusted with diatomaceous earth. 100 µl of a 0.42 µg/ml solution of tobacco mosaic virus ("TMV") was applied to the each dusted leaf. The TMV was gently and evenly spread across the leaves. Approximately 5 minutes after inoculation, the plants were lightly rinsed to remove the diatomaceous earth. The number of TMV lesions on the treated leaves was recorded five days after inoculation and is shown in Figure 4 below. *hrpN* transgenic plants had approximately 35% fewer TMV lesions than non-transgenic plant.

Figure 4. TMV Resistance in *hrpN* Transgenic vs. Wild Type Tobacco



31. Because disease resistance has been demonstrated for topical application of HrpN of *Erwinia amylovora*, HrpZ of *Pseudomonas syringae*, and HreX of *Xanthomonas campestris* (*see supra* at ¶¶ 18-20 and 22-24), and transgenic expression of *hrpN* of *Erwinia amylovora* and *hrpZ* of *Pseudomonas syringae* (*see supra* at ¶¶ 21 and 28-30), one of ordinary skill in the art would expect other members of this art-recognized class to likewise induce disease resistance in plants following topical application or transgenic expression thereof.

32. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 8/11/04

Zhong-Min Wei